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Effects Of De-Polymerization Of Cellulose In Producing Sugar From Cellulosic Biomass At Low-Temperature

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EFFECTS OF DE-POLYMERIZATION OF CELLULOSE IN PRODUCING SUGAR FROM CELLULOSIC BIOMASS AT LOW-TEMPERATURE

By

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By

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DEDICATION

This thesis is dedicated to my parents Ping Wu and Jinlan Xu, my husband Jinlong Li, my aunt Caiyun Zhang, as well as my grandparents for their unconditional support, love, and invaluable educational opportunities.

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Abstract

Lignocellulosic biomass is an abundant, renewable and environmentally friendly raw materials that can be converted into biofuels as an efficient alternative energy source. Sugar production is one of the key steps in converting biomass to fuels and chemicals. In order to enhance the accessibility of lignocellulosic biomass to catalytic enzyme, microorganism, and other types of catalysis during bioprocessing, the pre-treatment of lignocellulosic biomass is needed. To depolymerize the lignocellulosic materials, a 7% NaOH/12% urea solution was used. Selection of enzyme from Novozyme was performed, as a result, NS22074, a Cellulase complex was used throughout the research. Under the same reaction conditions, the treated cotton fibers were converted to glucose in almost 65% yield (based on 90% cellulose in cotton) within 2 hours at room temperature (22ºC); while the conversion for non-treated cotton fibers were only 20%. Different composition of NaOH/urea as the de-polymerization solutions were used and studied. Cellulose dissolution with NaOH/thiourea , LiOH/urea, KOH/urea were also used and studied. Next step is to apply the same pretreatment method to three different switchgrass samples, Alamo, Kanlow, and Bluegrass. Reaction time for swithgrass samples takes longer than that for cotton fibers, pretreated switchgrass samples were converted to glucose in 45% yield (based on 25% cellulose in switchgrass) within 3 days; non-treated swithgrass samples were only 20% yield.

Besides cotton fibers, Avicel® PH-101 commercial cellulose, printer paper, luffa, and a cotton t-shirt were also treated with this novel procedure. Acid hydrolysis using 10% HCl and 5% HCl was performed. The results showed that there is almost no glucose conversion present. Scanning electron microscopy (SEM) studies of the treated and non-treated samples have showed that the structure of treated sample is significantly different from the non-treated ones. High-performance liquid chromatography (HPLC) result showed that glucose is the only product produced. Fourier transform infrared spectrometry (FTIR) results showed a sharp peak in both 3400cm^{-1} and 1500 cm^{-1} region for pretreated samples, which explains less hydrogen bonding can allow urea hydrates self-assembled at the surface of the NaOH for enzymatic hydrolysis.

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LIST OF SYMBOLS OR ABBREVIATIONS

CBU .....Cellobiase Unit

EGU .....Endo-Glucanase Unit

FBG ..... Fungal β-Glucanase Unit

FXU-S ..... Fungal Xylanase Unit

AGU .....Amyloglucosidase Unit

PGU .....Polygalacturonase Unit

SEM .....Scanning Electron Micrscopy

FT-IR ..... Fourier Transform Infrared Spectroscopy

HPLC ..... High Performance Liquid Chromatography

CHAPTER I

INTRODUCTION

On Earth, there are two types of energy sources. Renewable energy is the energy that will naturally replenish on a human timescale such as sunlight, wind, waves and geothermal heat[1]. Non-renewable energy does not renew itself on a human timescale such as fossil fuels. It has been reported that many of the constituent states of the European Union are facing severe, critical shortages of fossil fuels and other natural resources in the near future[2]. With the rapid development, to create alternative renewable energy becomes a global concern. The need for alternative renewable energy is growing due to the climate change, energy security, economic benefits, oil prices, increasing population and decreasing in water[3]. Biofuel is a renewable fuel energy made from biomass. Bioethanol, a popular type of biofuel, is a promising alternative to reduce dependence on crude oil[4]. It has been used as transportation fuel since 1975 when the ProAlcool program, a national alcohol program, was started in Brazil to overcome the energy crisis. Since 1976, Brazil government made it mandatory to blend ethanol with gasoline for vehicles; and Brazil has become energy self-sufficient by producing bioethanol[5]. In 2011, Brazil and United States were responsible for 87.1% of the world's ethanol fuel production[6].

Lignocellulosic biomass is an abundant, renewable and environmental friendly raw material that can be converted into biofuels as an efficient alternative energy source[7]. Lignocellulosic biomass is composed of three principal components: cellulose, hemicelluloses, and lignin. The distribution of these components varies depending on the feedstock source; however, because of the rigidity of the cellulose chain, handling and molding the cellulose is very difficult. Cellulose belongs to neither thermoplastic nor thermosetting polymers, and is hardly soluble in general organic or inorganic solvents. Therefore, the difficulty of cellulose dissolution becomes one of the major limitations of its application. Development of better dissolving systems is special importance in cellulose industry and other fields.

There are number of approaches to produce regenerated cellulose, such as viscose rayon and Lyocell fibers, but the environmental concerns limited these applications. Effective cellulose solvents should be able to break down the cellulose chain into fragments; sodium hydroxide is the most popular one. NaOH solution can cause cellulose to swell and can even dissolve cellulose in a narrow range of the phase diagram. In recent years, Zhang's research group found that sodium hydroxide with urea at cold temperature can dissolve cellulose better than sodium hydroxide alone. They found 6%NaOH/ 4%urea, 7%NaOH/ 12%urea, and 6%NaOH/ 5%thiourea are good solvents for cellulose. The mechanism they proposed is that NaOH destroys the inter- and intra- hydrogen bonds between

cellulose molecules. Urea hydrates could be self-assembled at the surface of the NaOH hydrogen-bonded cellulose complex to form relatively stable inclusion complex at low temperature which led to dissolution of cellulose[8,9]. However, the lack of understanding of the NaOH and NaOH/urea dissolution process significantly constrains its applications.

The conversion of lignocellulosic biomass to ethanol involves a series of steps: pretreatment to enhance digestibility of biomass, enzymatic hydrolysis to break down the cellulose to glucose monomers, fermentation to convert the sugars into ethanol and distillation to remove from the ethanol[10]. In order to maximize the yield of the final product, ethanol, each of these steps must be optimized to achieve the maximum conversion rate. This thesis will focus on the first two steps in order to improve the potential yield of glucose for the other two steps. The pretreatment procedure and enzymatic hydrolysis steps will be discussed in further detail in later section.

In order to fully understand the cellulose dissolution in an alkali system, our focus in this study is to develop a novel procedure to produce glucose from cotton fibers, and then apply the method to switchgrass or other biomass material. The main objective of this project is to convert switchgrass samples into glucose that is useful for bioethanol production. In order to do so, pretreatment and enzymatic hydrolysis of the biomass will be performed. The following points will be the main focus of this research to achieve the main objective:

- Since each biomass substrate responds differently to pre-treatment methods, an appropriate pre-treatment method will be designed for switchgrass samples to de-polymerize the cellulose structure.

- The determination of an appropriate enzyme will allow for efficient hydrolysis and increased conversion of reducing sugar. The selection of an appropriate enzyme from Novozyme will be studied.

- In order to fully understand the cellulose dissolution in an alkali system, we choose cotton fibers to be our staring material. Then, the behavior of cotton with and without NaOH/urea at low temperature in alkali system will be studied. The studies of cotton fibers with NaOH/thiourea, LiOH/urea, KOH/urea and Na_2CO_3/urea will also be studied and discussed.

- Studies of cotton fibers with different compositions of NaOH/urea solution will be studied in this research. Based on the observation of the experiment, a possible cellulose dissolution mechanism will be proposed.

- Apply the proposed procedure to three different types of switchgrass, Alamo, Kanlow, and Bluegrass. The glucose yields of switchgrass samples will be studies, and also the study of the effects of

hemicelluloses and lignin with the bleaching chemicals will be present.

- Other cellulose materials, such as Avicel® PH-101 Microcrystalline cellulose, printer paper, luffa, 100% cotton t-shirt will be studied under the same reaction condition with the same novel procedure.

- Acid hydrolysis with 10% HCl and 5% HCl will be performed and studied.

- Sample studies using Scanning Electron Microscopy (SEM), High-Performance Liquid Chromatography (HPLC) and Fourier Transform Infrared Spectroscopy (FT-IR) will be involved and used in the studies of lignocellulosic biomass in this research

Research in conversion of biomass to ethanol are expensive, and with the limited commercialization process. This research intends to show that using our proposed novel procedure, high yields of glucose are possible and glucose is the only product produced. This would result in increased production efficiency of ethanol, which is a keystone to its commercialization.

1.1 Lignocellulosic Biomass

Lignocellulosic biomass is the most abundantly raw materials for the production of bioethanol. It refers to plant material that is composed of cellulose, hemicelluloses, and lignin. Nowadays, lignocellulosic material is highly interest to researchers in the field of biofuel and bioenergy. The composition of lignocellulosic biomass varies depending on different feedstock; a summarized data is listed in Table 1-1. In general, lignocellulosic biomass contains approximately 30-50% cellulose, 20-30% hemicelluloses, and 10-25% lignin[11-15].

Table 1-1: Lignocellulose Composition, Based on wt%[16-19]

	Cellulose %	Hemicelluloses %	Lignin %
Cotton seed hairs	80-95	5-20	-
Grasses	35-50	25-40	-
Paper	85-99	0	0-15
Newspaper	25-40	40-55	18-30
Corn cobs	45	35	15
Switchgrass	30-35	40-45	12
Hardwoods stems	25-40	45-47	20-25
Softwood stems	25-29	40-45	30-60

It has been estimated that lignocellulosic biomass could meet about 54% of the annual consumption of oil in the U.S. and that by 2030 biomass-based fuels could supply approximately 20% of the nation's transportation fuel market[20].

Cellulose is a polysaccharide with formula $(C_6H_{10}O_5)_n$, occurring principally in the cell walls of plants and fungi. This polysaccharide is created by the photosynthesis from carbon dioxide and water, using the sunlight as the energy source.

$$6CO_2 + 6H_2O \text{ (+ light energy)} \rightarrow C_6H_{12}O_6 + 6O_2$$

Cellulose consists of unbranched polymer within thousands β–D-glucose molecules. α-D-glucose and β–D - glucose are stereoisomer; they have the same molecular formula but differ in 3-dimensional configuration of atoms/groups at one or more positions. The carbon to the right of the oxygen atom in the hexagonal ring is called the anomeric carbon. If the –OH group attached to it is below the ring, the molecule is α-D-glucose. If the –OH group is above the ring, the molecule is β–D - glucose. Figure 1-1 is the schematic molecular structure of cellulose. The structural of the cellulose chain can be quite long; generally, 20-30 repeating units give all cellulose properties[21].

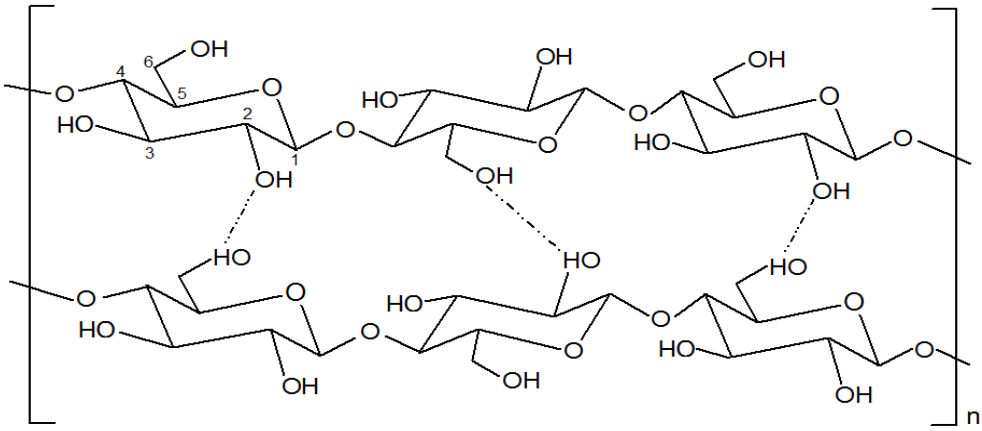


Figure 1-1: Molecular Structure of Cellulose

Each cellulose chain has two ends, one with an original C1-OH is called the reducing end and the other one with an original C4-OH group is called the non-reducing end[22]. The multiple hydroxyl groups on the glucose from one chain form hydrogen bonding with oxygen on the same or neighbor chain, holding the chains firmly side-by-side, also to form a rather straight linear chain. In fact, many chains can align in this manner, forming sheets. Furthermore, such a sheet can form hydrogen- bonded associations with other sheets in parallel. The extensive H-bond network that forms when the glycan chains align to form sheets and stack of sheets helps account for the rigid, tough characteristic of cellulose fiber.

Starch is a polymer of glucose where all the repeat units are directed in one direction and are connected by alpha linkages. Unlike the cellulose, starch can be safely eaten by humans because we have enzymes that can break it down into

glucose. Cellulose itself is a hydrophilic material. It can be very difficult to dissolve in aqueous solution due to the existing of large quantities of hydrogen bonds, which group cellulose chains together to form a network[23]. Strong intra- and inter-molecular hydrogen bonds in cellulose prevent its molecules from dissolution in most general inorganic and organic solvents.

Hemicelluloses are mixed polymers; it has a random and amorphous structure[24]. Figure 1-2 is the hemicelluloses structure[25].

In this polymer, mannose is the most important hemicellulosic monomer followed by xylose, glucose, galctose, arabinose and glucuronic acid. In comparison, cellulose and hemicelluloses can be quite different. Hemicelluloses are a mixed polymer, whereas cellulose is a polymer with just glucose.

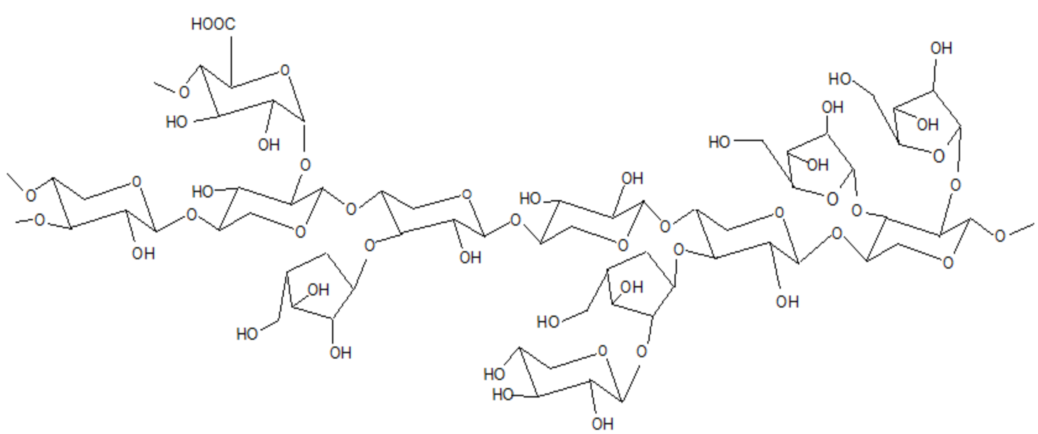


Figure 1-2: Hemicelluloses Structure

Cellulose has long unbranched polymer while hemicelluloses have short side-chains. Hemicelluloses have low molecular weight; however, cellulose has very high degree of polymerization. The solubility and susceptibility to hydrolysis of hemicelluloses are greater than cellulose. Table1-2 summarized the comparsion[26].

Table 1-2: Comparison of Cellulose and Hemicelluloses

	Cellulose	Hemicelluloses
Monomer	Only glucose	Mixed sugar
Polymer chain length	Long (5m)	Short
M.W.	High (10000 units)	Low (hundred units)
Polymer topology	Unbranched	Branched
Side groups substitution	No substitution	On C2, C3, and C6
Polymer morphology	Crystalline + amorphous	Amorphous
Solubility	Low	High
Reactivity	Less reactive	More reactive
Hydrolysis	Partial	Readily (susceptible)

Lignin is a constituent of the plant cell walls; it is the second most abundant natural polymer in the world[27]. Lignin has the unique characteristic due to the large-scale of an aromatic functionality. As shown in Figure 1-3, lignin is

composed of mainly three monomers, coniferyl alcohol (A), sinapyl alcohol (B), and p- coumaryl alcohol (C)[28].

Lignin held cellulose and hemicelluloses together to provide stiffness and rigidity. It prevents the absorption of water by these polysaccharides in plant cell walls and allows the efficient transport of water in the vascular tissues.

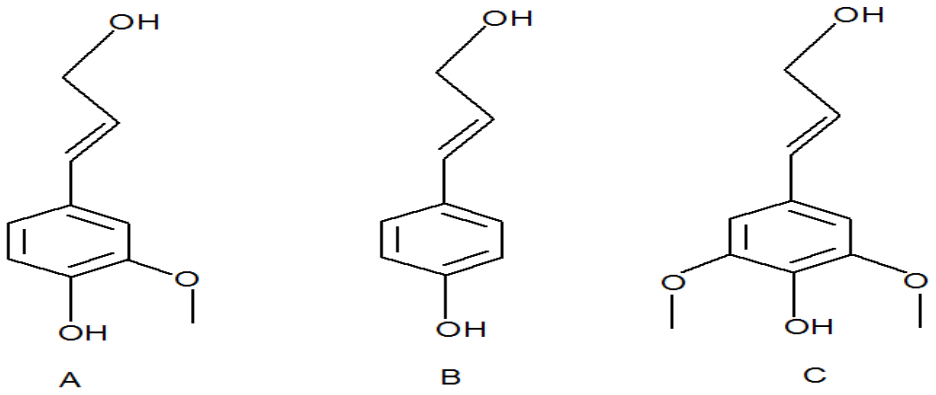


Figure 1-3: Coniferyl Alcohol, Sinaply Alcohol, and p-Coumaryl Alcohol

1.2 Pretreatment Methods

The main goal of pretreatment is to make the biomass more susceptible for enzymatic attack, therefore increasing the conversion rates and sugar yields. Pretreatment breaks apart the bonds that hold the cellulose, hemicelluloses and lignin together. Figure 1-4 illustrates the effect of pretreatment of lignocelluloses structure[29].

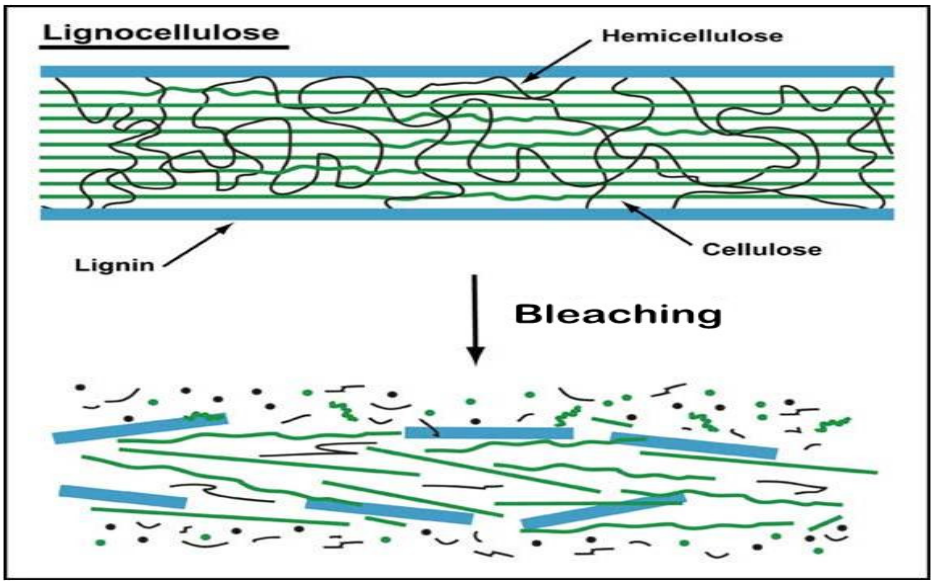
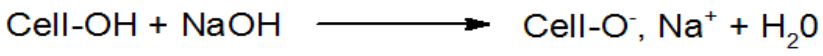


Figure 1-4: Effect of Pretreatment on Lignocelluloses Structure

Now we have the basic knowledge about cellulose structure, let's take a brief review of some pretreatment methods. Pretreatments such as thermochemical, stem explosion, ammonia fiber explosion, liquid-hot-water treatment, and oxidative process can all lead to physically disruption of biomass fiber and partially decrystallization of cellulose, which are the basic requirements for sugar production chemically or enzymatically[30-34]. However, all of these pretreatments require a large amount of energy can lead to sugar degradation. For industry purposes, these are not very helpful. With all of these pretreatments require either high temperature or high pressure or both. There are number of approaches to produce regenerated cellulose at low temperature. This part we focus on the pretreatment methods at low temperature and take a brief review of each cellulose pretreatment methods.

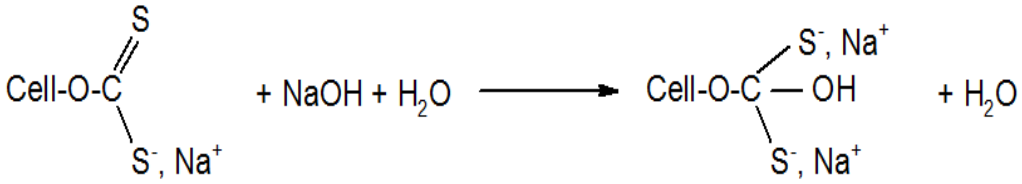
Viscose Method, Viscose was first created by a French scientist Hilaire de Chardonnet. Viscose is a solution of cellulose xanthate made by treating dissolving pulp with sodium hydroxide and carbon disulfide. Viscose rayon fiber is regenerated cellulose and it has similar structure to cotton. To prepare viscose, the process can be divided into three major steps: alkali activation, xanthation and dissolution[35].

First, cellulose fibers from spruce, pine, or other cellulosic materials need to be soaked in 17-20% NaOH solution at room temperature for a few hours to convert native cellulose into alkali-cellulose.



Cellulose fibers are swollen in this process and activated for other chemical treatment. After removing extra NaOH solvents by pressing, the alkali cellulose further need to be shredded into small pieces and aged under controlled temperature for almost two days. During aging, cellulose will be degraded into desired degree of polymerization that determines the viscosity of viscose solution. Then the alkali cellulose will be mixed with carbon disulfide for xanthation for hours.

The resulting cellulose derivative is cellulose xanthate, which is soluble in dilute NaOH solution (under 6%).



Although this viscose process has been industrialized for a long time, the hazardous chemicals (mainly CS_2 and H_2S) released from this process are a huge environmental problem that cannot be solved simply by optimization and improvement of the viscose process. Alternative method is needed to replace this process.

Lyocell Process, Lyocell is a form of rayon, developed and first manufactured in the 1980s. A process with a new solvent system based on N-methylmorpholine N-oxide monohydrate (NMMO). NMMO is a 'direct' solvent of cellulose which means it can dissolve without prior activation or derivatization. NMMO is well known as an oxidizing agent in organic chemistry. It can dissolve

cellulose due to its strong N-O dipole. The dissolution procedure is much simpler comparing with the viscose process. First, about 8~23% of conventional cellulose fibers are dispersed into 50% of NMMO in water to make a slurry, then this suspension is concentrated at higher temperature (60°C) at reduced air pressure until NMMO monohydrate is made (with water content of 13.3% and melting point of 74°C). This monohydrate is proved to be a better solvent for cellulose than pure NMMO. After dissolution, the cellulose water solution can be used for fiber and film formation through different processes[36].

The chemistry of the NMMO/cellulose system is very complex; the process is hard to control due to the multiple reaction pathways in the system. Since there are still lots of problems with viscose process and lyocell process, in recent years, researchers are still working to find the cellulose solvents; NaOH is the most popular one.

NaOH- Water Solution, Sodium hydroxide is a common strong base and is the most popular one among these processes. It is largely used in the pulp and paper industry. The concentration we are interested in this study is in the range of 0~10%.

A phase diagram of cellulose-NaOH-water system plotted by Sobue is shown in Figure 1-5[37]. With different NaOH concentration and temperature combination, cellulose would interact with NaOH in various different ways to form

different complex. In particular, NaOH can cause cellulose to swell and can even dissolve cellulose in a narrow range of the phase diagram. In small triangle region in Figure 1-5, the NaOH concentration is between 6% - 10% and temperature is from -10℃ to 4℃. Cellulose is highly swollen in this region. Later on, researchers found that NaOH can be a direct solvent of cellulose in this region.

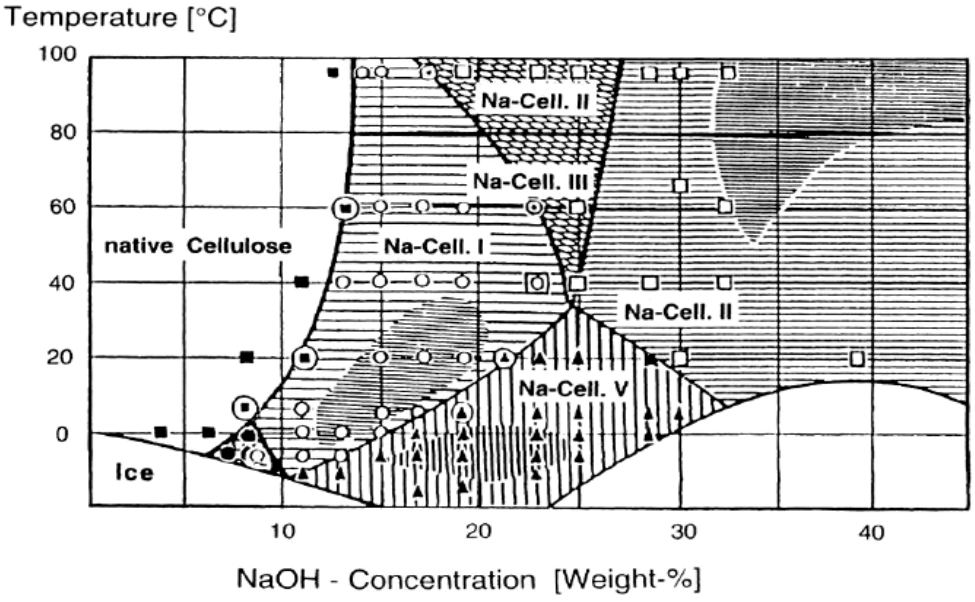


Figure 1-5: Phase Diagram of Cellulose-NaOH-water System

NaOH with Urea Solution Process, In recent years, Zhang's research group performed many investigations on cellulose dissolution in NaOH/urea solution and their applications. They found that certain concentration of NaOH/urea and NaOH/thiourea are good solvents for cellulose dissolution. The recommended

compositions are 6%NaOH/4%urea, 7%NaOH/12%urea and 6%NaOH/5%urea. They explained the mechanism is due to the urea hydrates can self-assembled at the surface of the NaOH hydrogen-boned cellulose complex to form relatively stable inclusion complex at low temperature. Urea hydrates function as hydrogen bonds donor and receptor between solvent molecules and prevents the re-association of cellulose molecules which leading to dissolution of cellulose[38,39].

CHAPTER II

EXPERIMENTAL

2.1 Materials and Chemicals

Cotton fibers and Avicel® PH-101 microcrystalline cellulose were chosen as the starting material and tested at the beginning of this research. Cotton fibers were selected as the starting material is due to the composition of cotton contains 80-90% cellulose, 5-20% hemicelluloses, and 0% lignin. Avicel® PH-101 microcrystalline cellulose is available in lab, and it is a high purity cellulose powders for partition chromatography[40]. Since microcrystalline cellulose particle size is approximately 50μm; it is really hard to filter during the filtration step. After few experiments, we eliminated Avicel® PH-101 microcrystalline cellulose due to the particle size and also the structure difference. Details will be discussed in later section.

Printer paper, luffa, 100% cotton t-shirt, are used and tested with proposed novel procedure. The main focus of the second part study is to produce glucose from switchgrass samples. Three different types of switchgrass samples are studied; they are Alamo switchgrass, Bluegrass switchgrass, and Kanlow switchgrass. All switchgrass samples are from the Agriculture department at EKU. Urea and thiourea in crystal form, and NaOH, LiOH, Na_2CO_3, HCl, $NaClO_2$, CH_3COONa are all purchased from Aldrich. Avicel® PH-101 microcrystalline cellulose is purchased form Sigma-Aldrich. Cotton balls are purchased from supermarket.

2.2 Experimental Setup

Pretreatment of lignocelluloses, which can break the seal of lignin and hemicelluloses, reduce the cellulose crystallinity, and increase the enzymatic accessibility of cellulose[41,42]. For cotton fiber samples, lignin is not a problem. This helps us to design the low temperature pretreatment method without worry the by-product that produced from lignin. For the second part of the study, a bleaching step is added before the low temperature pretreatment method to break down the coat of lignin[43].

The cellulose pretreatment kinetics was studied under a series of low temperature from 0ºC to -10ºC. The design of the low temperature reactor requires low cost and high efficiency. The design included a commercial cooler as a cooling bath, in this case, ethylene glycol is used in the commercial cooler; a temperature control system that can set for low temperature (Figure 2-1). Ethylene glycol, or ethane-1,2-diol disrupts hydrogen bonding when dissolved in water. Pure ethylene glycol freezes at about -12˚C, but when it mixed with water, the mixture does not readily crystallize, and the freezing point of the mixture depressed. In the commercial cooler with ethylene glycol present, it helps us to depolymerize the cotton fibers in a constant temperature at -10˚C.

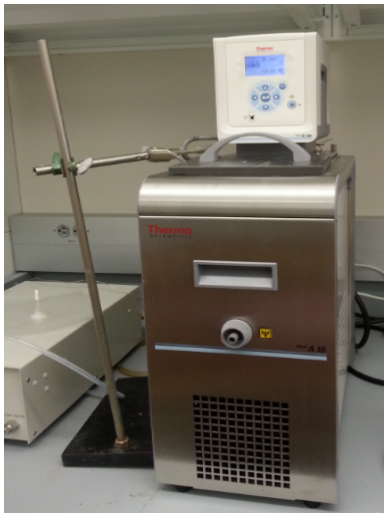


Figure 2-1: Commercial Cooler Used in Lab

Alkali solutions will be cooled at -10ºC, -6ºC, -3ºC and 0ºC, then placed the weighted samples (~0.1280mg) with stirring for 4mins. After that, the solution was placed and stayed at room temperature (22ºC). After it reached room temperature, removed the samples from the solution by filtration, and washed them with deionized water for at least six times, so it reached a neutral pH. Once we obtained the washed samples, we then added the buffer solution (0.1M sodium acetate and 0.1M acetic acid) and the enzyme NS22074, the Cellulase complex. Let it develop and tested the results periodically with Benedict's test.

On the other hand, after pretreatment, we filtered out the samples from solution and washed with de-ionized water for at least six times and then dried under vacuum for scanning electron microcopy (SEM) images (Figure 2-2).

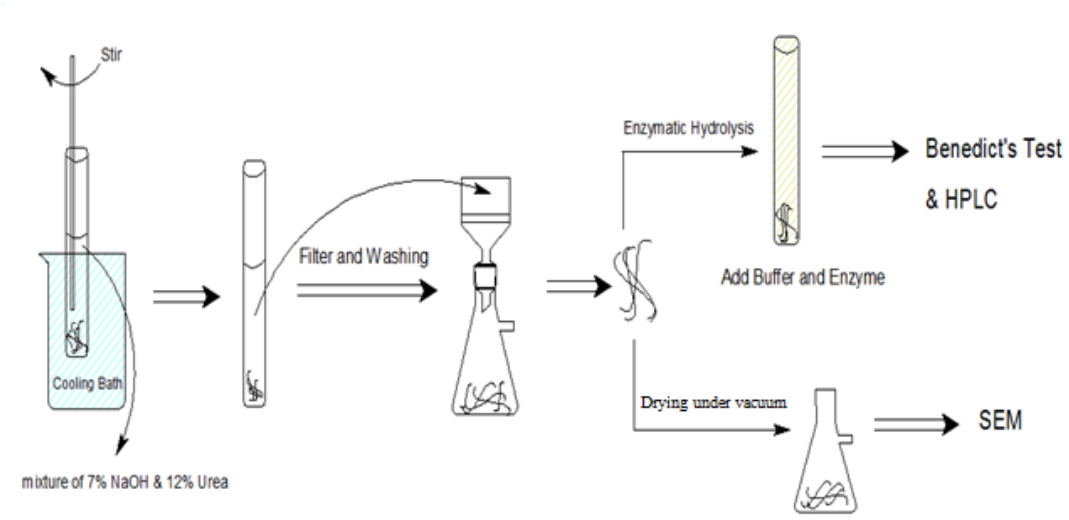


Figure 2-2: Flow Chart of Proposed Production Method for Glucose

For switchgrass samples, a bleaching step is added before the proposed production method for glucose (Figure 2-3). The darkness color of the switchgrass is caused by lignin; a bleaching step is very necessary to remove the lignin contents by using bleaching chemicals sodium chlorite and sodium acetate[44]. Bleaching chemicals can break down the lignin molecule, disrupt lignin-carbohydrate bonds, and allow the fragments to dissolve. Followed with the pretreatment method, enzyme can then react with cellulose and produce sugar. After the bleaching step, all switchgrass samples or other biomass materials will follow the exact same procedure just like the steps that were done using cotton fibers. Please refer to Figure 2-3 and Figure 2-4 for detailed steps.

Step 1:

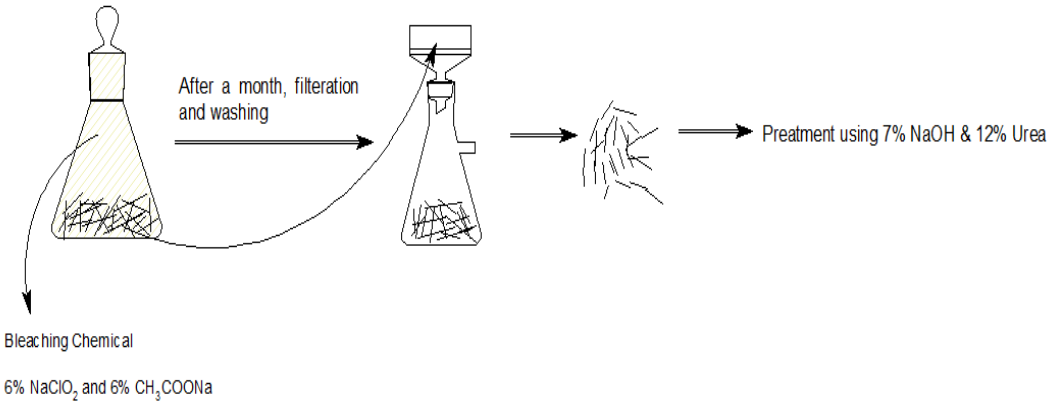


Figure 2-3: Bleaching Step

Step 2:

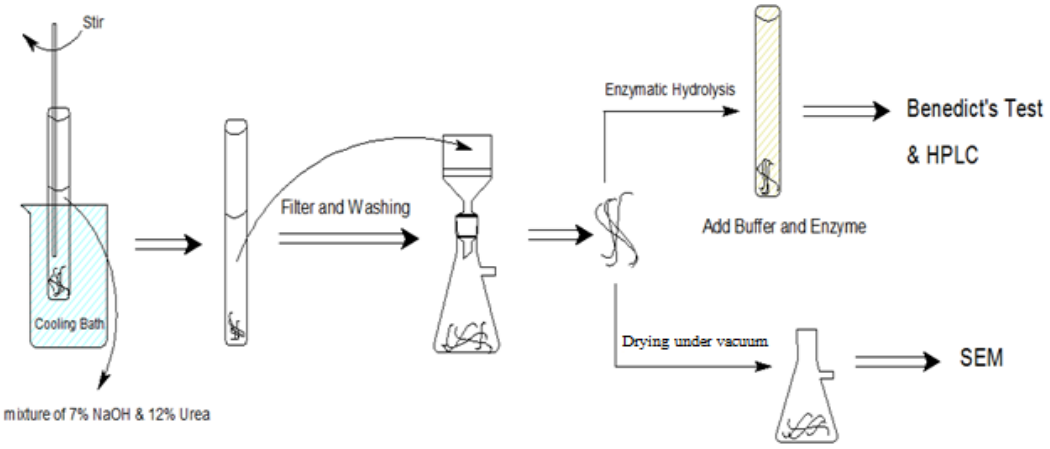


Figure 2-4: Flow Chart of Proposed Production Method for Switchgrass

2.3 Quantitative Determination of Glucose

Benedict's test is used to determine the quantities of glucose; it is named after an American chemist, Stanley Rossiter Benedict[45]. It is commonly used for the presence of reducing sugar(s); includes all monosaccharides and many disaccharides. Sugars are classified as reducing or non-reducing based on their ability to act a reducing agent during the Benedict's test. Sugars that contain aldehyde groups are oxidized into carboxylic acid. Please see Figure 2-5 for the reaction.

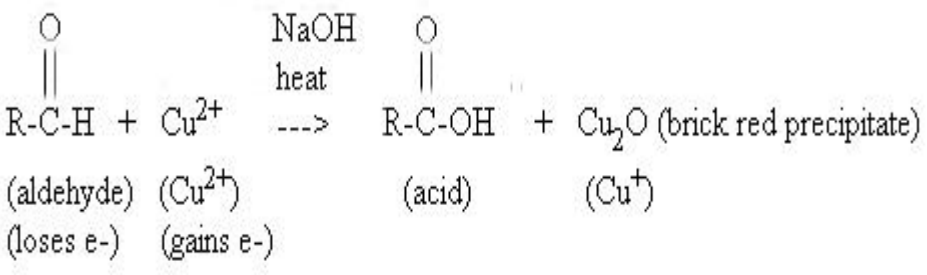


Figure 2-5: The Principle of Benedict's Test[46]

When the reducing sugars are heated in the presence of an alkali, they converted into powerful reducing compounds known as enediols. Enediols reduce the cupric ions (Cu^{2+}) present in the Benedict's reagent to cuprous ions (Cu^{+}), which get precipitated as insoluble red copper (I) oxide[47]. The color of the obtained precipitate gives an idea about the quantity of sugar present in the solution.

Depending on the quantity of reducing sugar present, the color of the mixture will vary. Benedict's test have its limitations, it will detect reducing sugar not only in glucose, and also in other sugars, such as fructose and maltose. Benedict's test cannot be used to determine the reducing sugar in sucrose.

Below pictures give you a better understanding of how color changed in the Benedict's test (Figure 2-7, 2-8, 2-9, and 2-10).

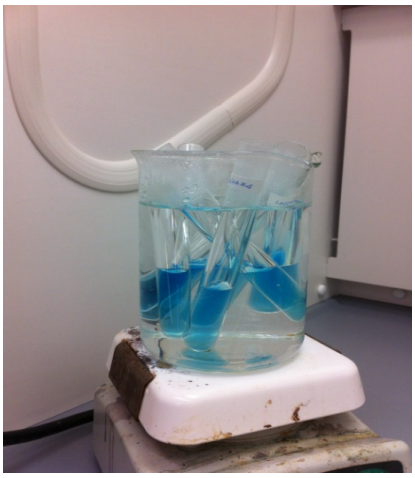


Figure 2-6: Samples with Benedict's Solution

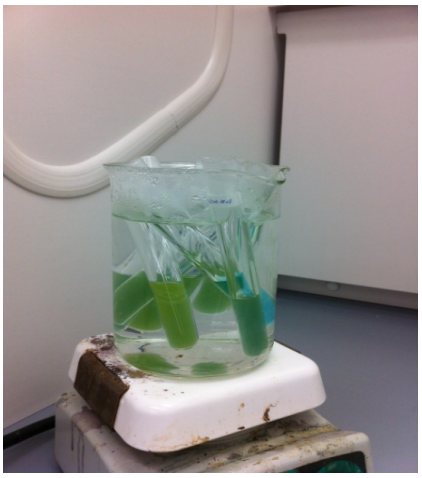


Figure 2-7: Heating after 2mins with Benedict's Solution

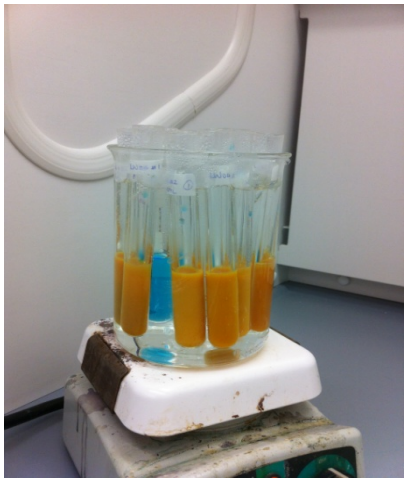


Figure 2-8: Samples with Benedict's Solution Show Positive Result (after 10mins)

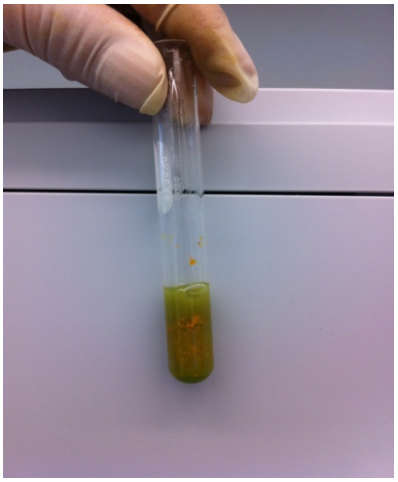


Figure 2-9: Samples with Glucose Presents

2.4 Enzymatic Hydrolysis of Cellulose

In order to break down the cellulose structure, hydrolysis reaction by enzyme is needed. After pretreatment, the biomass is available for enzymatic attack. When enzyme is added to the biomass, it acts as a catalyst to break down the bonds for the polysaccharides.

The enzymes we used in this study are provided from Novozymes. There are six different enzymes, NS22074 (cellulase complex), NS50010 (β-glucosidase), NS50012 (enzyme complex), NS22002 (β-glucanase xylanase), NS22035 (glucoamylase), NS22036 (xylanase). The selection enzyme and the determination of the enzyme volume have to be made based on the yields of sugars. To start with,

we chose NS22074 cellulase complex, it is the primary enzyme for the use in the hydrolysis of lignocellulosic materials. NS22074 will break down cellulose in the pretreated material into glucose and cellobise[48]. Then we tested NS22074 in different volumes, from 0.1ml~1.0ml. As results, under the same reaction condition, 0.4ml enzyme volume produces the highest yields of sugar.

Next, we examined all six enzymes with the same enzyme volume, 0.4ml. As result, NS22074 produces the highest yields of sugar.

A summarized data is in Table 2-1. Please note that the company Novozyme provides data such as activity, density, pH and temperature labeled as company data. The experimental data is the examinations performed in the lab. Descriptions of enzymes contained in the Novozymes biomass kit are included in the appendix.

Table 2-1: Enzyme Activity, Density, pH, Temperature, and Yield

	Company Data				Experimental Data
Enzyme Classification	Activity	Density g/mL	pH	Temperature ℃	Yield
NS22074 Cellulase complex	1,000 EGU/g	1.15	5.0-5.5	45-50	82%
NS50010 β-glucosidase	250 CBU/g	1.2	2.5-6.5	45-70	19%

Table 2-1 (Continued)

	Company Data				Experimental Data
Enzyme Classification	Activity	Density g/mL	pH	Temperature (˚C)	Yield
NS50012 Enzyme Complex	100 FBG/g (~13,700 PGU/g)	1.19	4.5-6.0	25-55	76%
NS22002 β-glucanase xylanase	45 FBG/g (~470 FXU/g)	1.20	5.0-6.5	40-60	39%
NS 22035 Glucoamylase	750 AGU/g	1.15	4.5-5.5	60-70	63%
NS 22036 Xylanase	1,000 FXU-S/g	1.09	4.5-6.0	35-55	18%
NS50012 Enzyme Complex	100 FBG/g (~13,700 PGU/g)	1.19	4.5-6.0	25-55	76%

2.5 Calibration Curve

In analytical chemistry, a calibration curve is a graph showing the value of some property versus concentration of analyte. When the corresponding property of an unknown is measured, its concentration can be determined from the graph[49]. Solutions containing known concentrations of analyte are called standard solution.

Solutions containing all the reagents and solvents used in the analysis, but no deliberately add analyte, are called blank solution[50].

The data, the concentration of the analyte and the instrument response for each standard can be fit to a straight line, using linear regression analysis. This can be described by equation $y= mx+y_o$, y is the instrument response, m represents the sensitivity, y_o is a constant that describes background. The analyte concentration x of an unknown samples can be calculated from this equation.

The standard calibration curve in this study was done with known glucose samples, run with Biotek Synergy 2 UV-vis plate reader (Figure 2-10). Y-axis represents the absorbance, X- axis represents concentration. It measured the Cu^{2+} value corresponding to absorbance; it is an indirect relationship.

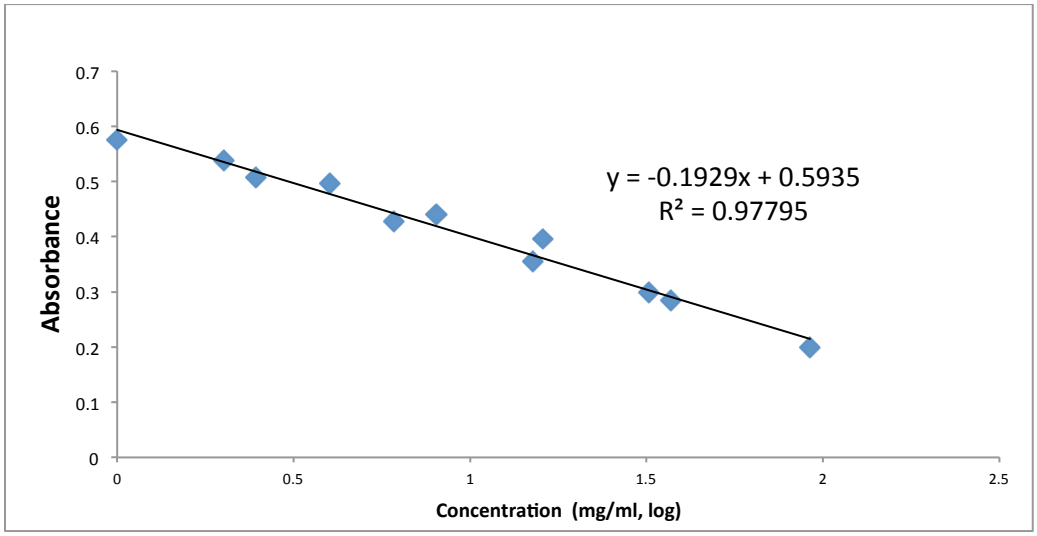


Figure 2-10: Standard Calibration Curve for Glucose

CHAPTER III

PRETREATMENT WITH DIFFERENT BASE/UREA

The alkali metal are group in the periodic table consisting of the chemical elements lithium (Li), sodium (Na), potassium (K), rubidium (Rb), caesium (Cs), and francium (Fr). The alkali metals have very similar properties: shiny, soft, highly reactive metals at standard temperature and pressure[51]. Because of these properties, we want to test cotton fibers in different alkali solutions at low temperature to see if they perform similar results, same as the NaOH/urea.

3.1 Results and Discussion

Under the same reaction condition and followed by exact same procedure with cotton fibers in different alkali solutions. We used Zhang's research group suggested pretreatment composition, 7% NaOH/ 12% urea to begin with. Pretreating cotton fiber with Na_2CO_3/urea, KOH/urea and LiOH/urea at -10ºC for 4 minus, the results are summarized in Table 3-1.

Pretreatment with Na_2CO_3/urea, KOH/urea and LiOH/urea did not depolymerized the cotton fibers. Figure 3-1 is a picture of cotton fiber before the pretreatment in a cooled 7%NaOH/12%urea at -10ºC solution.

Table 3-1: Observations of Cotton Fibers in Different Alkali Solutions

	Temperature (ºC)	Results
18% Na_2CO_3/ 12%Urea	-10	Not dissolving
4.2% LiOH/ 12%Urea	-10	Not dissolving
9.8% KOH/ 12%Urea	-10	Not dissolving
7% NaOH/ 12%Urea	-10	Depolymerized

In fact, the cotton fibers pretreated with Na_2CO_3/urea, KOH/urea and LiOH/urea appeared gritty texture first (Figure 3-2); and then, it went back to the original state after when it reached room temperature at 22ºC (Figure 3-3).The pretreatment with NaOH/urea depolymerized the cotton fibers and appeared almost clear solution (Figure 3-4).

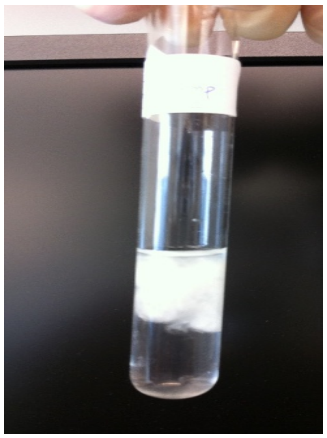


Figure 3-1: Cotton Fibers before Pretreatment

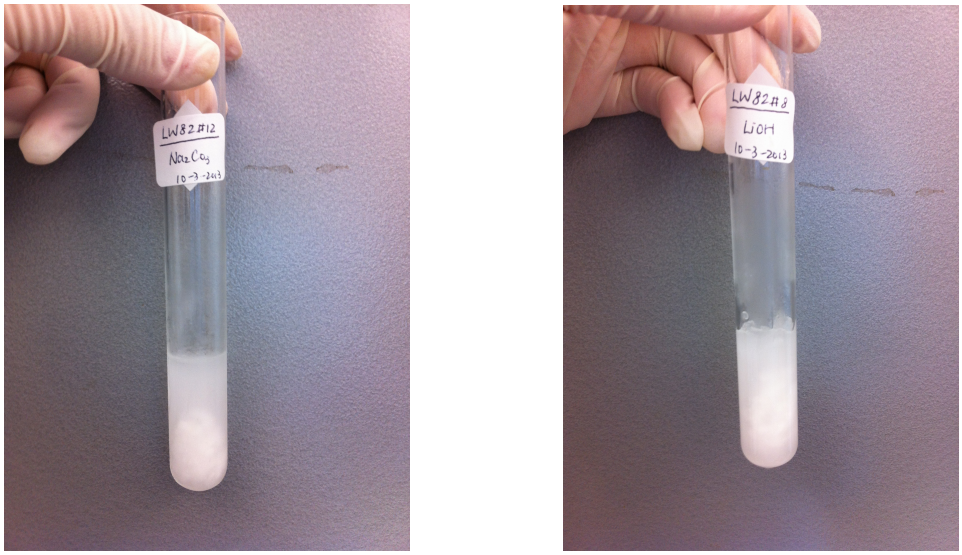


Figure 3-2: Pretreatment with Na_2CO_3/urea (L) and LiOH/urea (R) at -10ºC for 4mins

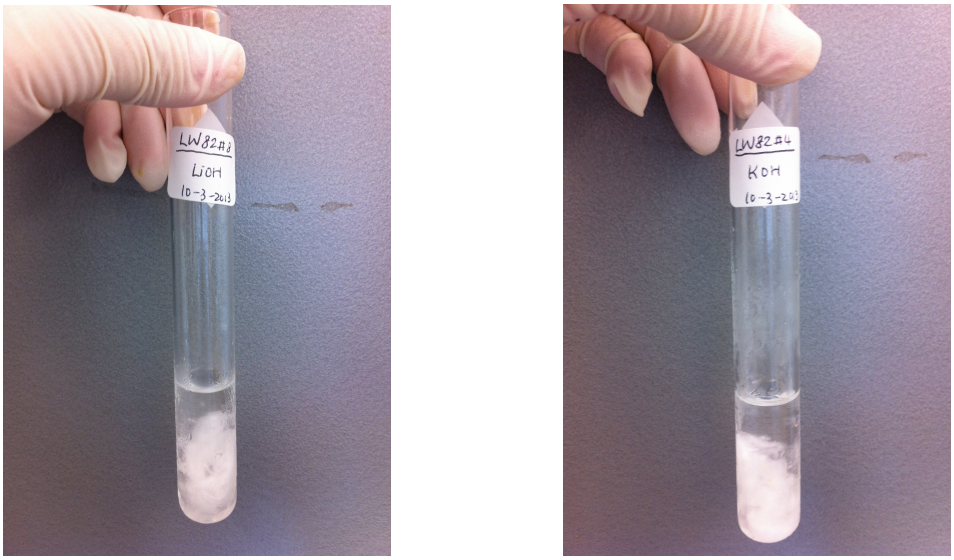


Figure 3-3: LiOH/urea (L) and KOH/urea (R) after pretreating When it Reached Room Temperature (22ºC)

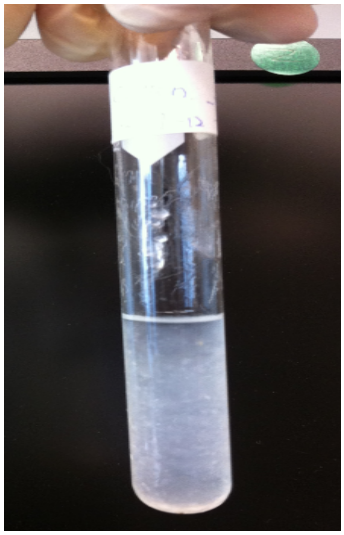


Figure 3-4: Pretreatment with NaOH/urea at -10ºC for 4mins

Even though only NaOH/urea depolymerized cotton fiber, but may be the other alkali solutions did some effect to the cotton fibers and it was just cannot be observed by naked eyes. Therefore, I still followed the same procedure with all pretreated cotton fibers in different alkali solutions. After pretreatment, washed all treated cotton fibers with deionized water for at least six times and tested with pH paper for a neutral pH. Then added 0.4ml NS22074 Cellulase complex. Tested each samples in every 30mins within two hours using Benedict's test.

Na_2CO_3/urea results were not included because the glucose production reached over 100% after the first 30mins and it reached even higher after two hours. LiOH/urea and KOH/urea have little or almost no glucose production since the yields of glucose are lower than 25% and 20%. However, NaOH/urea reached almost 70% yields after two hours. A summarized data is in Table 3-2.

Table 3-2: Enzymatic Hydrolysis Comparison of LiOH/urea, KOH/urea and NaOH/urea

Solution	30mins	60mins	90mins	120mins
LiOH/Urea	18%	21%	24%	22%
KOH/Urea	16%	15%	17%	15%
NaOH/Urea	28%	44%	60%	68%

For treated cotton fibers with 7%NaOH/ 12%urea, the cotton fiber almost all disappeared (Figure 3-5). However, since the yields of glucose for LiOH/urea and KOH/urea is very low, there are no change to the cotton fibers. One reason we think is due to the ionic radius. Ionic radius increase as descending a group, in our case, Li^+ has the smallest ionic radius, 0.076nm; Na^+ has a ionic radius of 0.102nm, while K^+ has the highest ionic radius of 0.138nm (Table 3-3)[52]. The activity coefficient for Na^+ is 0.90, K^+ is 0.64. Na^+ is the right size to fit in or even break the inter-cellulose chain which leads to sugar production.

Table 3-3: Ionic Radius of Each Ion

	Ionic Radius
Li^+	0.076nm
Na^+	0.102nm
K^+	0.138nm

Other reason is because 7% NaOH is soluble in water at -10ºC, the solubility for LiOH and KOH decreases, as the temperature gets higher. Therefore, they are not as basic any more. Pure water freezes at 32ºF (OºC), with NaOH, the freezing point decreases. However, for LiOH and KOH the freezing point will be different. LiOH and KOH cannot be use as pretreatment solution at -10ºC, they have already became a solid.

Figure 3-5: Initial Observation of Glucose Results using 7%NaOH/12%urea at -10ºC

3.2 Pretreating Cotton Fibers with 7%NaOH/12%Thiourea at -10ºC

Thiourea is structurally similar to urea, except the oxygen atom is replaced by a sulfur atom (Figure 3-6)[53].

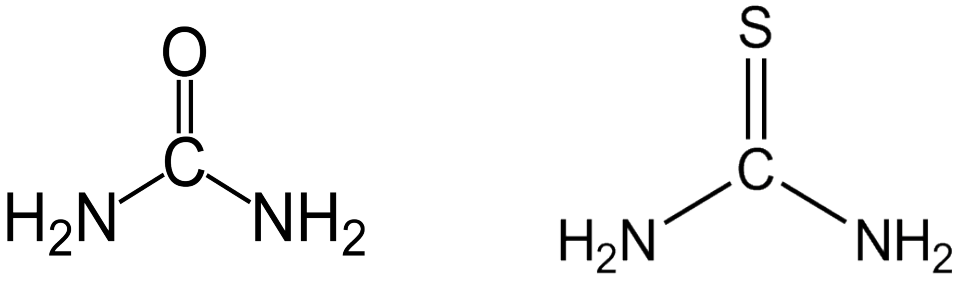


Figure 3-6: Structure Comparison of Urea (L) and Thiourea (R)

Because of the structure similarity, and also Zhang's research group obtained NaOH/urea and NaOH/thiourea aqueous solution which can dissolve cellulose directly and quickly[54,55]. We decided to pretreated cotton fibers using 7%NaOH/15.2 % thiourea with the exact same procedure. After two hours, it reached almost 70% yield. As conclusion, because of the structure similarity, thiourea can be used as an alternative for urea to pretreat lignocellulosic materials.

Working with thiourea has to be extremely careful, avoid all skin contacts, avoid inhalation, wear face shield, and it's combustible.

CHAPTER IV

PRETREATMENT OF COTTON WITH NaOH/UREA AT LOW-TEMPERATURE

Now we know pretreating cotton fiber with 7%NaOH/12%urea at -10ºC gives us good results. We want to study this alkali solution systematically. This chapter is focusing on pretreating cotton fibers at different temperature, 0ºC, -3ºC, -6ºC, and -10ºC with different NaOH concentration, 7% NaOH/12%urea, 6%NaOH/12%urea, 5%NaOH/12%urea. After that, compared the yields of glucose for each concentration, to see whether if there is a trend when lower the concentration of NaOH, the percent yields of glucose maybe will decrease. Then, examine the non-treated and treated cotton fibers using the scanning electron microscopy (SEM) and fourier transform infrared spectroscopy (FT-IR) for the structure change. And also examine the product after two hours using high performance liquid chromatography (HPLC) to detect whether there is any glucose produced for a confirmation test.

4.1 Results and Discussion

When pretreating cotton fibers with 5%NaOH/12%urea at -10ºC, -6ºC, -3ºC and 0ºC, the cotton fibers is not depolymerized observed by naked eyes (Figure 4-1). As matter of fact, there is almost no change to the cotton fibers when using

5%NaOH/12%urea for the pretreatment, which means the cellulose structure is not broken or disrupted, leads to no or low yields of glucose. On the other hand, pretreatment with 6%NaOH/12%urea experience very similar results comparing with pretreatment with 5%NaOH/12%urea.

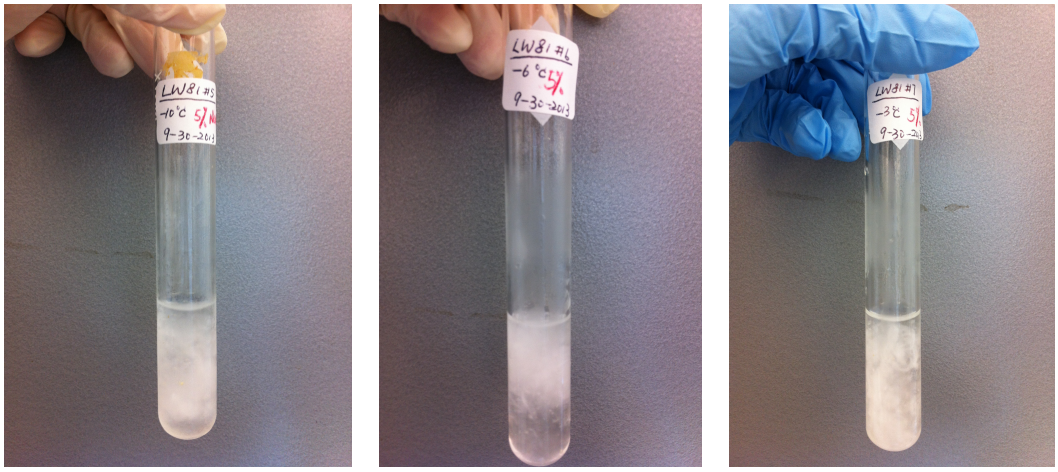


Figure 4-1: Pretreating Cotton Fiber with 5%NaOH/12%urea at -10ºC(L), -6ºC(M), and -3ºC(R)

Table 4-1 lists the results for pretreated cotton fibers with 7%NaOH/12%urea at different temperature. As results, when pretreating at -10ºC, it has the highest yield of glucose, almost reaching 65% within two hours.

Table 4-1: Use 7%NaOH/12%urea Pretreat Cotton Fibers in Different Temperature

Temperature (ºC)	Glucose Production (%)	
	Room Temperature (22ºC)	Water Bath (35ºC)
0	16.85	13.79
-3	40.56	50.30
-6	54.89	62.51
-10	54.69	62.35

The yields of glucose decrease as the temperature increase. Enzymatic hydrolysis took place at two different temperature setting, one is in room temperature at 22ºC, and another one is in water bath at 35ºC. Temperature affects the yield of glucose; however, without the raise of the temperature, the reaction can still take place. The yield of glucose for 7%NaOH/12%urea at -10ºC reached 55% within two hours in room temperature (22ºC). It is very close to the one that is done in water bath (35ºC), reached 62%. Pretreated cotton fibers with 7%NaOH/12%urea at 0ºC has less than 20% glucose yield, we then decided to neglect the pretreatment at 0ºC.

Systematically studies of pretreated cotton fibers using 7%NaOH/12%urea were done within two hours, and the results are tested in each 20 minutes. Since

there is not a huge difference whether the enzyme is developed in room temperature or in water bath, after the enzyme NS22074 Cellalse complex is added, we decided to let it develop under room temperature at 22ºC. Summarized results are in Table 4-2.

Table 4-2: Studies of Pretreated Cotton Fibers with 7%NaOH/12%urea in each 20mins

Solution	**Temperature (ºC)**	**Glucose production**					
		20 mins	40 mins	60 mins	80 mins	100 mins	120 mins
7% NaOH/ 12% urea	-10	28.32%	34.40%	47.27%	54.96%	65.96%	65.37%
	-6	25.47%	27.96%	36.96%	47.32%	53.35%	50.96%
	-3	10.24%	13.08%	15.34%	25.14%	41.45%	39.67%

Enzymatic hydrolysis can be run at room temperature is very essential to this research. Saving energy is the key and we are trying to minimize the cost of each step. Clearly, pretreated cotton fibers with 7%NaOH/12%urea at -10ºC have the highest glucose yield. After two hours, pretreated cotton fibers with 7%NaOH/12%urea at -6ºC reached 51% yield, and for pretreatment at -3ºC, it reached almost 40% yield. Next, we changed the concentration of NaOH, and followed the exact same procedure, and tested the pretreated cotton fibers with

6%NaOH/12%urea and 5%NaOH/12%urea at different temperature in each 30 minutes within two hours. Summarized results are in Table 4-3.

Table 4-3: Studies of Pretreated Cotton Fibers with 6%NaOH/12%urea and 5%NaOH/12%urea in each 30mins

Solution	**Temperature (ºC)**	**Glucose production**			
		30 mins	60 mins	90 mins	120 mins
6%NaOH/12%urea	-10	24.73%	27.64%	29.73%	30.71%
	-6	18.44%	20.31%	22.29%	25.39%
	-3	17.20%	15.62%	15.33%	17.90%
5%NaOH/12%urea	-10	5.32%	7.07%	8.18%	8.12%
	-6	4.44%	5.60%	8.05%	7.76%
	-3	2.85%	3.04%	4.20%	5.24%

The yields of glucose reduced by almost half when using 6%NaOH/12%uera as the cellulose dissolution; and the percent yield is even lower for pretreating with 5%NaOH/12%urea. The percent yield decreases as the composition of NaOH decreases. These results correspond to the phase diagram of the cellulose-NaOH-water system. In the small triangle region, NaOH concentration is between 6% to 10%; temperature is from -10ºC to 4ºC, NaOH can be a direct solvent of cellulose and pretreatment with 7%NaOH/12%urea at -10ºC

is the most suitable condition for cellulose materials and gives the highest yields of glucose.

SEM is referring to scanning electron microscope, is a type of electron microscope focused beam of high-energy electrons to generate a variety of signals at the surface of solid specimens[56]. Accelerated electrons in an SEM carry significant amounts of kinetic energy, and this energy is dissipated as a variety of signals produced by electron-sample interactions when the incident electrons are decelerated in the solid sample. The SEM is used to generate high-resolution images of shapes of objects and to show spatial variations in chemical compositions[57].

After pretreatment with 7%NaOH/12%urea at -10ºC for 4 minutes, wash cotton fibers for at least six times with deionized water. When the pH is neutral, placed two or three cotton fibers in a clean vial and dried under vacuum for at least three days. First, the dried non-treated and treated cotton fibers were tested using a compound microscopy (Figure 4-2) under 400 magnifications. Non-treated cotton fiber is twisted, and treated cotton fiber appears to be a straight linear line.

After this, we knew there is a change in cellulose structure, and we want to look the fibers closely in a larger magnification using SEM. The SEM used in this research is the NeoScope JCM-5000 benchtop Scanning Electron Microscope. Images are provided in Figure 4-3 and 4-4 for both non-treated and treated cotton fibers under 6000 magnification. The images of SEM showed that the surface of

the non-treated cotton fiber appears straight, linear line, and it's very smooth; however, treated cotton fiber with 7%NaOH/ 12%urea at -10ºC for 4mins appears something looks like bubbles on the surface. This change leads us to believe that after pretreatment the surface of the cotton fibers have already been damaged and it is readily available for enzymatic hydrolysis.

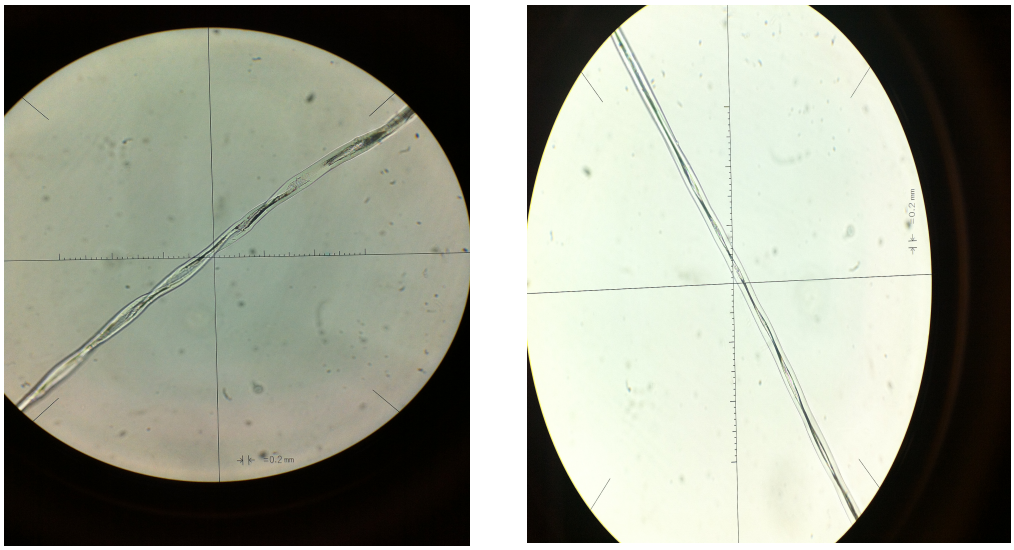


Figure 4-2: Non-treated Cotton Fiber (L) and Treated Cotton Fiber (R) using Compound Miscroscopy under 400x

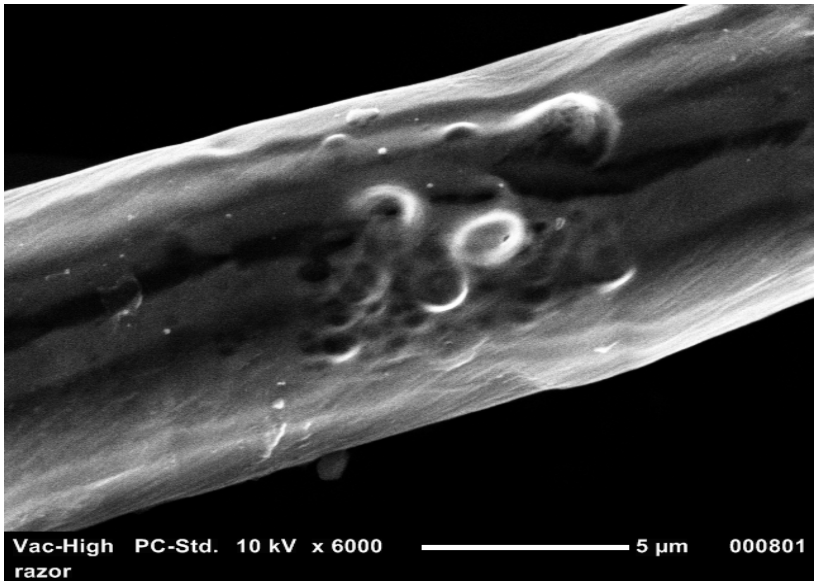


Figure 4-3: SEM Image of non-treated Cotton Fiber under 6000X

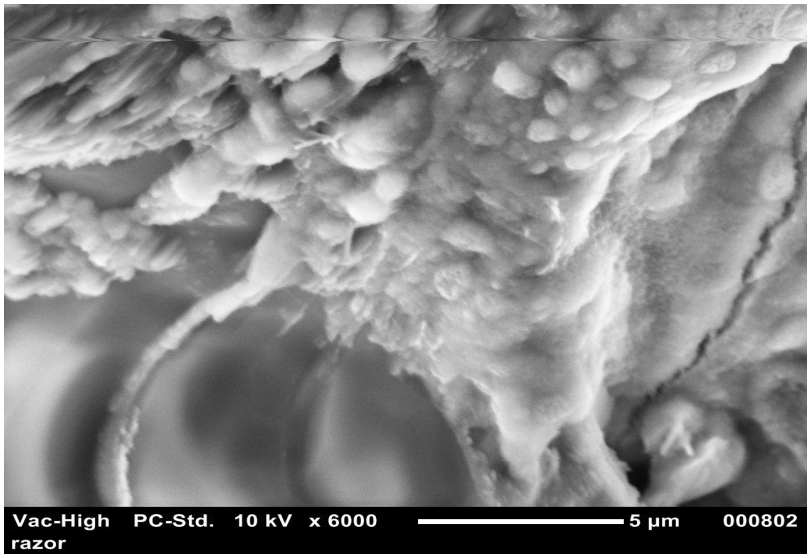


Figure 4-4: SEM image of Treated Cotton Fiber under 6000x using 7%NaOH/12%urea at -10˚C for 4mins

High performance liquid chromatography or high pressure liquid chromatography (HPLC) is a technique of column chromatography used in analytical chemistry to separate the components in a mixture, identify each component and quantify each component. HPLC utilizes a column that holds chromatographic packing materials (stationary phase), a pump that moves times of the molecules. Retention time varies depending on the interactions between the stationary phase, molecules being analyzed, and the solvents used[58]. In this research, we used a Dionex® Ultimate 3000 HPLC system in normal phase with amniopropyl column [Phenomenex, 5µm, 250mm × 4.6mm] using a isocratic mobile phase consisting of 80:20 acetonitrile, water was delivered at a flow rate of 1.5mL/min. Solutions were injected into the HPLC system through the Ultimate® 3000 auto sampler. Detection was achieved with a Shodex R101 refractive index. In most cases, ultraviolet detector is often used for HPLC. However because of the UV range for glucose is very low; in this case, refractive index detector is used.

Figure 4-5 is the chromatography for blank sample. Blank sample is a sample containing all components except analyte. Figure 4-6 shows that glucose is the only product produced.

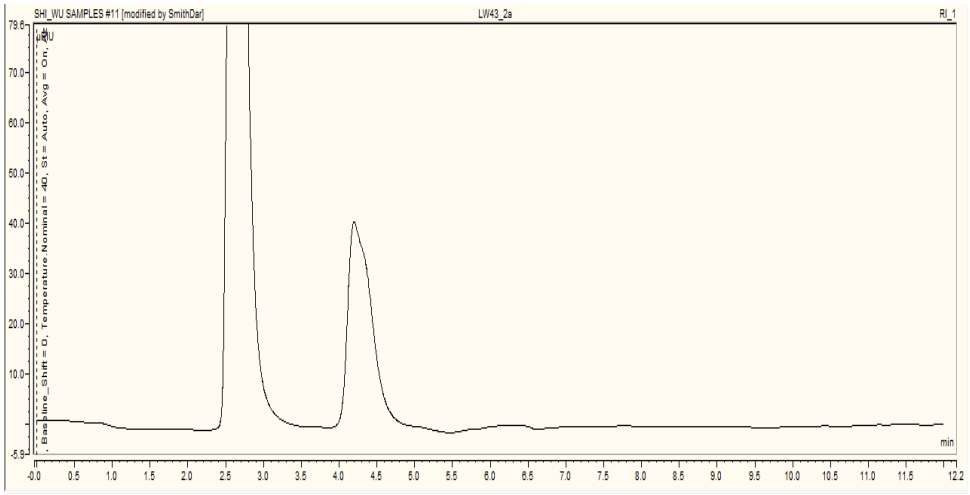


Figure 4-5: Chromatography of Blank Sample

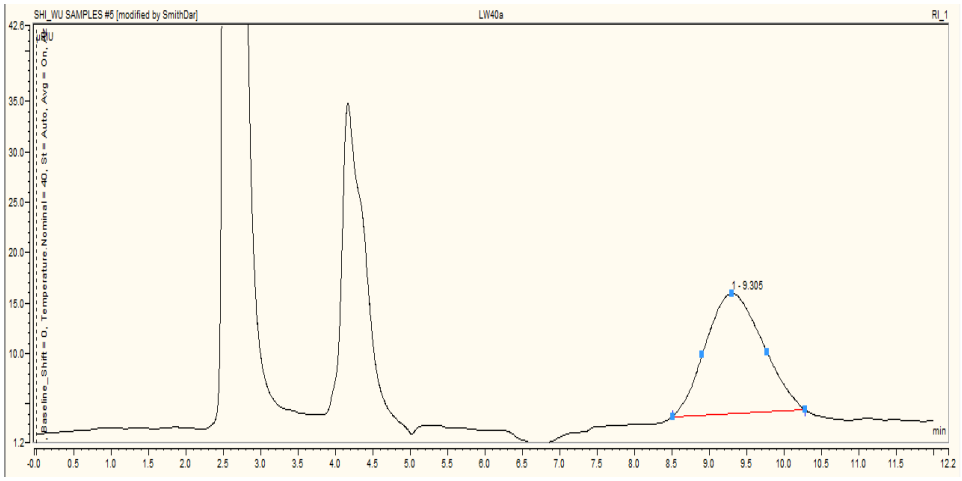


Figure 4-6: Chromatography of Treated Cotton Fiber with 7%NaOH/ 12%urea at -10ºC for 4mins

CHAPTER V

PRETREATMENT WITH SWITCHGRASS SAMPLES USING 7%NaOH/12%UREA AT -10˚C

The second part of this research is to apply the novel procedure that we obtained from the first part using pretreatment with 7%NaOH/12%urea at -10˚C to three different switchgrass samples, Alamo, Kanlow, and Bluegrass. Since all switchgrass samples contain some amount of lignin. The first thing to do is to remove lignin, if not, with lignin holding cellulose and hemicelluloses together. Enzymatic hydrolysis cannot break down the cellulose into fragments. Bleaching means removing or altering the color substances, the darkness color of switchgrass is caused by lignin. If the lignin is removed, the switchgrass samples appear lighter color. Bleaching chemicals are oxidizing agents that break down the lignin molecule, introduce solubilizing groups into the fragments, and disrupt lignin-carbohydrate bonds, allowing fragments to dissolve[59]. Sodium chlorite and sodium acetate (30 g/500ml sodium chlorite and 30 g/500ml sodium acetate) were used as bleaching chemicals; soaked the weighted switchgrass samples for a month.

$$2NaClO_2 + CH_3COONa \rightarrow 2NaCl + NaHCO_3 + CO_2 + H_2O$$

5.1 Results and Discussion

Alamo and Kanlow are a lowland switchgrass, is a warm-season perennial bunchgrass native to the Unites States[60]. All switchgrass samples were gifted from

EKU Agriculture Department. Figure 5-1 is the Alamo switchgrass before bleaching, it appears dark yellow color. Figure 5-2 is the Alamo switchgrass after bleached appears white color. This means, almost all lignin has been removed from switchgrass.

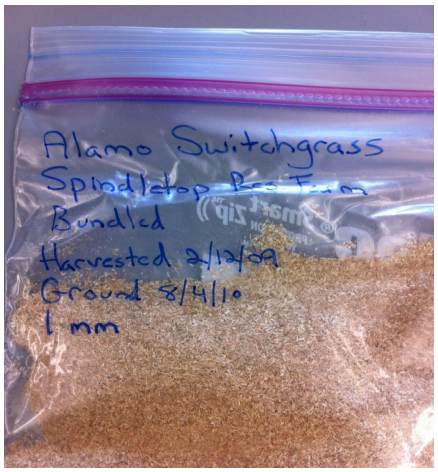


Figure 5-1: Alamo Switchgrass before Bleaching

Figure 5-2: Alamo Switchgrass after Bleached

After bleaching for a month, pretreated the switchgrass samples with 7%NaOH/12%urea at -10ºC for 4mins, and then left it at room temperature (22ºC). Washed the switchgrass samples with deionized water for at least six times, added enzyme NS22074 Cellulase Complex. The reaction time for switchgrass samples takes much longer than cotton fibers. Figure 5-3 is the observation for Alamo switchgrass samples, almost all samples are dissolved. Kanlow and Bluegrass switchgrass show similar results.

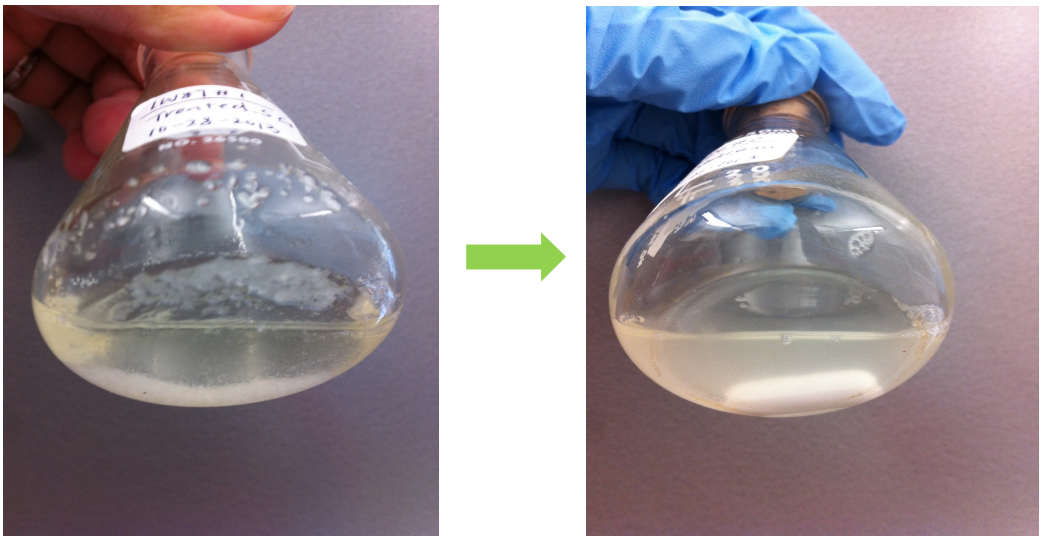


Figure 5-3: Observation of Treated Alamo Switchgrass Samples using 7%NaOH/12%urea at -10ºC for 4mins

Alamo switchgrass sample studies are summarized in Table 5-1. It showed that switchgrass samples take much longer time comparing with cotton fiber samples.

Table 5-1: Yields of Pretreated Alamo Switchgrass Samples

		Glucose Production				
Alamo Switchgrass		12hrs	24hrs (Day 1)	38hrs	45hrs	72hrs (Day 3)
	Treated	11.99%	26.25%	31.33%	35.68%	42.59%

Non-treated Alamo switchgrass sample reached 34% yield after three days. And for treated Alamo swithgrass samples, three days later it reached almost 45%. I kept examining the samples using Benedict's test on day 7 and day 9, the percent yield for glucose did not change much. On day 9, it reached 44.8% yield.

All three switchgrass samples Alamo, Kanlow, and Bluegrass have very similar results. Table 5-2 summarized the data .Alamo switchgrass samples have the highest glucose conversion, reaching almost 45% in 9 days. Kanlow and Bluegrass samples have a little lower conversion. Lignin percent is calculated after soaked all samples for a month using bleaching chemicals, sodium chlorite and sodium acetate. Then, washed them using deionized water for at least six times, and dried all samples in a sample vial under vacuum. After the sample is dry, weight the dried samples.

Table 5-2: Yields of Glucose for Switchgrass Samples and Calculated Lignin% Bleached

Type of Switchgrass	**Glucose Production**			**Calculated Lignin % Bleached out**
	Non-treated (without bleaching or pretreatment)	Day 3 (Bleached and Pretreated)	Day 9 (Bleached and Pretreated)	
Alamo	25.9%	42.6%	44.8%	22.2%
Kanlow	20.8%	35.4%	37.8%	8.7%
Bluegrass	21.2%	34.9%	38.2%	18.2%

And then to calculate, use the sample weight before bleaching minus the weight after bleaching; calculate the lignin percentage that might be bleached out during the bleaching process.

After added enzyme NS20074, the samples were developed in two different setting, one is in room temperature at 22ºC, and another one is in water bath at 35ºC. As result, this two different temperature setting did not affect the yield of glucose. In fact, the results are almost identical.

Scanning electron microscopy (SEM) can gives us more in depth the structure changes for our switchgrass samples. Non-treated switchgrass sample

means sample is bleached but before pretreatment; treated switchgrass sample means sample were bleached and treated using 7%NaOH/12%urea at -10ºC. Figure 5-4 and 5-5 are Alamo switchgrass samples bleached before pretreatment and bleached after pretreatment.

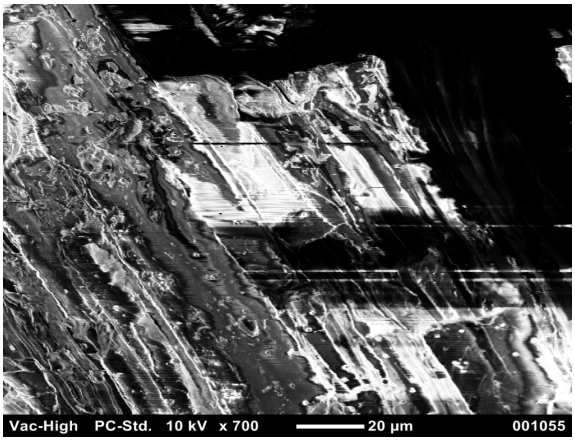


Figure 5-4: Bleached Alamo Switchgrass before Pretreatment at 700x

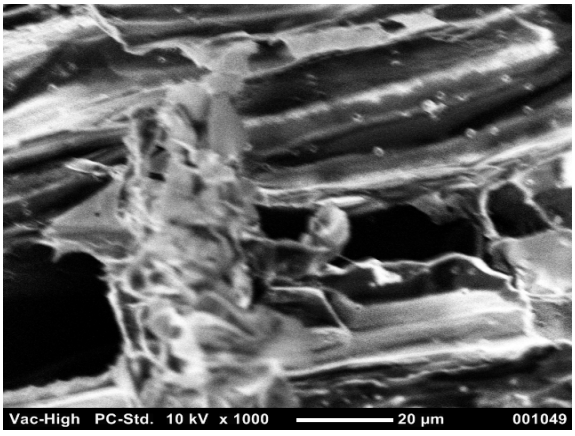


Figure 5-5: Bleached Alamo Switchgrass after Pretreatment at 1000x

Figure 5-6 and 5-7 are Kanlow switchgrass samples bleached before pretreatment and bleached after pretreatment.

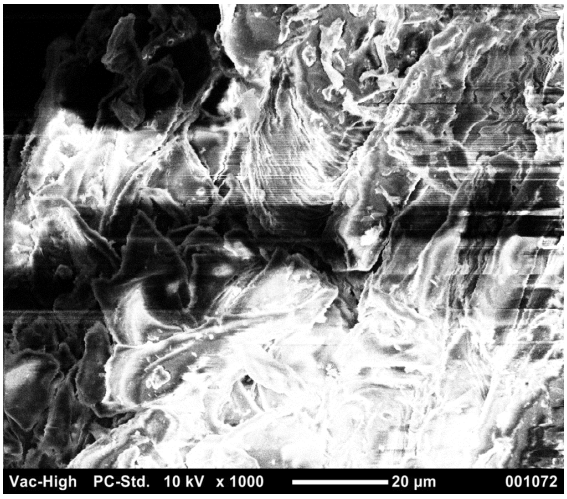


Figure 5-6: Bleached Kanlow Switchgrass before Pretreatment at 1000x

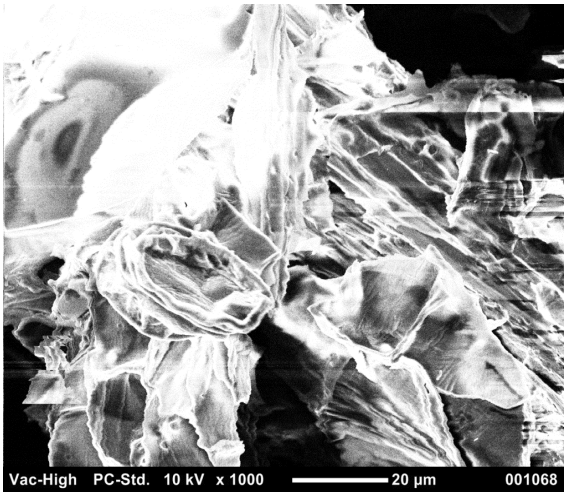


Figure 5-7: Bleached Kanlow Switchgrass after Pretreatment at 1000x

Figure 5-8 and 5-9 are Bluegrass switchgrass samples bleached before pretreatment and bleached after pretreatment.

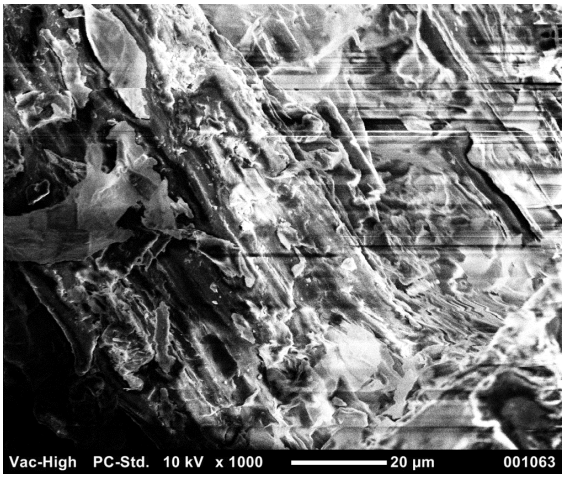


Figure 5-8: Bleached Bluegrass Switchgrass before Pretreatment at 1000x

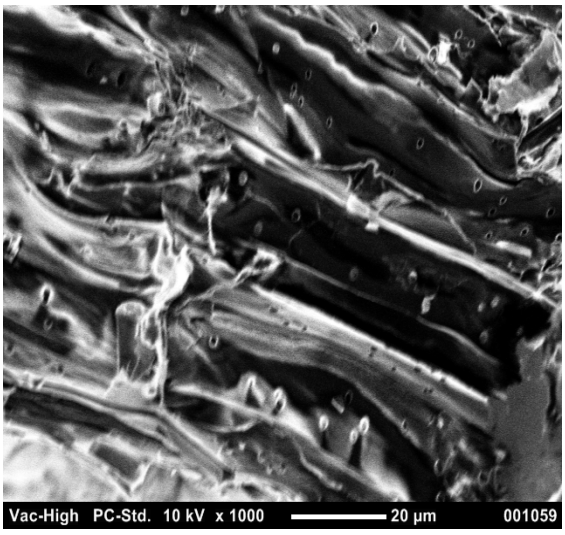


Figure 5-9: Bleached Bluegrass Switchgrass after Pretreatment at 1000x

As results, the SEM images of Alamo, Kanlow, and Bluegrass samples were not very clear. Scanning electron microscopy has large magnification; however, I can't take a clear image of switchgrass samples beyound1000 magnification. Even at the 1000 magnification, the images are not clear. When using the SEM, the tape where you place the sample will cancel the charges. In our case, we think there are still some charges that are on the sample which will cause the unclear images. Maybe this is due to the bleaching, since cotton fibers behavior well under SEM at 6000 magnification after pretreatment. Switchgrass samples are more complicated and need further investigation.

CHAPTER VI

PRETREATMENT WITH OTHER BIOMASS MATERIALS

A proposed production method for depolymerization of cellulose fibers have been designed using cotton fibers pretreated with 7%NaOH/12%urea at -10ºC. In this case, we believe pretreatment with other cellulose materials can also produce sugar by using this method. Other cellulose materials were tested in this chapter including Avicel® PH-101 microcrystalline cellulose, printer paper, luffa, and 100% cotton t-shirt. Also, hydrolysis of 10% and 5%HCl will be discussed in this chapter.

6.1 Avicel® PH-101 Microcrystalline Cellulose

In 1962, Battista and Smith reported a product named "Avicel" cellulose to the American Viscose Company of micrystalline cellulose. The "PH" designation indicated that the product is suitable for pharmaceutical use[61]. Avicel® PH-101 microcrystalline cellulose is purchased from Sigma-Aldrich. Follow the extract same procedure, pretreated cellulose with 7%NaOH/ 12%urea at -10ºC for 4mins. Since the microcrystalline cellulose is a fine particle in a powder form. Filtration after the pretreatment step was very difficult. Figure 6-1 is a great picture to explain this matter. Glucose residues are strung together in β(1-4) glycosidic linkages for cellulose; since this Avicel® PH-101 microcrystalline cellulose remains in alpha-

cellulose, some as starch, waters and other solvents can very easily to get in and break down into fragments. It means pretreating Avicel® PH-101 microcrystalline cellulose is not necessary. Therefore, we removed this cellulose from our pretreating biomass materials list.

Meanwhile, pretreatment of Avicel® PH-101 microcrystalline cellulose with 7%NaOH/12%thiourea was performed. Like the pretreatment with 7%NaOH/12%urea, due to the particle size of the microcrystalline cellulose, it is almost impossible to determine the glucose percent yields.

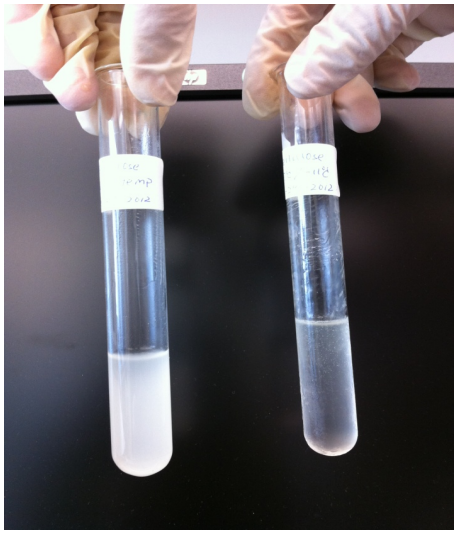


Figure 6-1: Non-treated (L), Treated (R) Avicel® PH-101 Microcrystalline Cellulose with 7%NaOH/12%urea at -10ºC

6.2 Printer Paper

We used printer paper as the starting material; followed with the exact same procedure, pretreated printer paper with 7%NaOH/12%urea at -10ºC for 4mins, and then compared the results with non-treated printer paper (without pretreatment, printer paper in buffer and enzyme solution). For both non-treated and treated printer papers, the paper almost all dissolved by using NS22074 cellulase complex enzyme (Figure 6-2 and 6-3). The chemical composition of paper will depends on the type or grade of paper. Typically most grades of paper consist of organic and inorganic materials. For printer paper, it contains 70-100% organic portion, which is the cellulose, hemicelluloses, and lignin. Only 0-30% is the inorganic portion, such as calcium carbonate, clay, and titanium oxide[62]. The cellulose in printer paper could be in strung together in α (1-4) glycosidic bond, which means pretreatment is not needed for enzymatic hydrolysis.

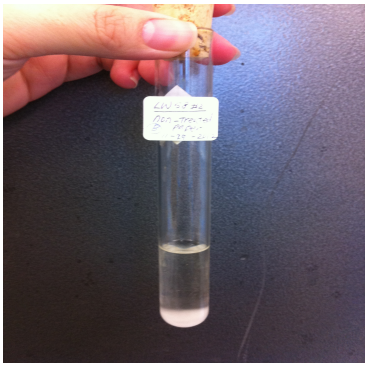


Figure 6-2: Non-treated Printer Paper

Figure 6-3: Treated Printer Paper with 7%NaOH/12%urea at -10ºC

NS22074 Cellulase complex is the first enzyme tested for enzymatic hydrolysis of printer paper. Since the results were not what we expected, we then used other five enzymes, NS50010 β-glucosidase, NS50012 Enzyme complex, NS22002 β-glucanase xylanase, NS22035 Glucoamylase and NS22036 Xylanase to examine the printer paper and to see if there are any changes. A summarized data is in Table 6-1.

Table 6-1: Summarized Data for Enzymatic Hydrolysis of Printer Paper

Enzyme Type	**Condition**	
	Non-treated **(in buffer and enzyme solution)**	**Treated**
NS22074 Cellulase complex	Dissolved	Dissolved
NS50012 Enzyme complex	Partially Dissolved	Dissolved
NS22002 β-glucanase xylanase	Partially Dissolved	Dissolved
NS22035 Glucoamylase	Undissolved	Partially Dissolved
NS22036 Xylanase	Undissolved	Partially Dissolved
NS50010 β-glucosidase	Undissolved	Partially Dissolved

Under the same reaction conditions, pretreated all printer papers with 7%NaOH/12%urea at -10ºC for 4mins. For non-treated printer paper, the samples with NS50012 and NS22002 are partially dissolved. For treated printer paper,

except the NS50012 and NS22002 are dissolved; others are partially dissolved for samples that added with NS22035, NS22036, and NS50010. Glucose productions for printer papers using all six enzymes were also carried out; however, the results were not what we expected, and it needs further investigation.

Below in Figure 6-4 and Figure 6-5 shows a better understanding of what it means of printer paper dissolved or partially dissolved in the solution after added different types of enzyme.

Figure 6-4: Non-treated Printer Papers with Different Enzymes

Figure 6-5: Treated Printer Paper with Different Enzymes

During the process of paper making, the structure of cellulose and others has already been changed or rearranged, which will affect the results differently. Also, some of the enzymes contain glucose that means it is very difficult to determine what percentage of glucose produced from the paper, and which is from the enzyme itself.

6.3 Luffa

Luffa or Loofah or vegetable sponge, is a popular vegetable in China and Vietnam, and is a member of the cucumber family. Luffa fiber shares similar structure with other biomass materials, it contains 55-90% cellulose, 8-22% hemicelluloses, and 10-23% lignin. The chemical composition of luffa fibers can be

affected by different factors, such as weather condition, plant origin, etc[63, 64]. First, bleached the luffa fibers and soaked using sodium chlorite and sodium acetate for three days. Then after washed the luffa fibers with deionized water for at least six times (Figure 6-6), pretreated luffa fibers with 7%NaOH/12%urea at -10ºC for 4mins and then followed the exact same procedure. Few days later, nothing happened, luffa fibers did not dissolved or disappeared, still appears in the bottom of the test tube (Figure 6-7), and there was almost no glucose conversion.

Figure 6-6: Bleached Luffa Fibers

Figure 6-7: Treated Luffa Fibers with 7%NaOH/12%urea at -10ºC for 4mins

6.4 Cotton T-shirt

A piece of 100% cotton t-shirt was pretreated using 7%NaOH/12%urea at -10ºC for 10mins. After 10mins, nothing changed to the cotton t-shirt (Figure 6-8). I

also bleached the cotton T-shirt using bleaching chemicals, sodium chlorite and sodium acetate. Again, after three days, there is no change to the T-shirt. This could due to the process of t-shirt making, the structure of cellulose have been already changed. It is difficult to pretreat the samples with the same dissolution solvents using NaOH/urea.

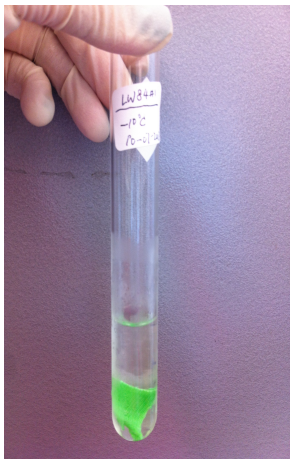


Figure 6-8: Treated Cotton T-shirt with 7%NaOH/12%urea at -10ºC

6.5 Hydrolysis with 10% and 5% HCl

10% and 5% HCl were prepared and used to replace the NS22074 cellulase complex for hydrolysis. Followed the exact same procedure, pretreated cotton fibers with 7%NaOH/12%urea at -10ºC for 4mins, then let it sit and reach room temperature (22ºC). After it reached room temperature, wash the cotton fibers with deionized water for at least six times; when it reached a neutral pH, remove the water, and add 10% HCl or 5 % HCl for hydrolysis. When tested the samples

periodically, the percent yield of glucose is only 24% when using 10% HCl hydrolysis. For hydrolysis using 5% HCl, there was almost no glucose produced. And it did not reach higher than that. Acid hydrolysis may not need the pretreatment step, otherwise it will not high glucose yield.

Chapter VII

CONCLUSION AND FUTURE WORK

A procedure for low-temperature pre-treatment of lignocellulosic materials was proposed. The work performed in the present thesis showed that pretreatment with 7%NaOH/ 12%urea at -10ºC is the best reaction condition for cellulose dissolution. Enzymatic conversion of cellulose to glucose can be done at room temperature by using this method. Pretreated cotton fibers were converted to glucose in almost 65% yield (based on 90% cellulose in cotton) within two hours. Bleaching chemicals sodium chlorite and sodium acetate can remove the lignin. Reaction time for swithgrass sample takes longer when comparing with cotton fiber samples. Switchgrass samples were converted to glucose in 45% yield (based on 25% cellulose in switchgrass) within three days. NaOH/thiouea can be used as a substitute for cellulose dissolution.

In the future, pH has to be monitored at each step during bleaching and pretreating switchgrass samples. Switchgrass samples are much more complicated than cotton fibers, monitor the pH at each step will benefits the further studies. The required enzyme dosage may vary significantly based on the specific composition of biomass feedstock. In the future, the studies of different enzyme dosage should be performed with Alamo, Kanlow, Bluegrass or other switchgrass samples. In order to maximize the yield from enzyme hydrolysis, a combination of enzyme

activities must be considered. According to the Novozyme company data, they recommended NS22074 cellulase complex paired with NS50010 β–glucosidase or NS22036 xylanase to maximize performance. Also, for practical applications the optimal conditions for NS22074 is about 45-50ºC (113-140ºF). A better temperature control system is needed to achieve better results for switchgrass samples. SEM images of switchgrss sample without bleaching and without pretreatment need to be taken. Many other effects are also important but not included in this study for the time limit, such as, the origin of switchgrass samples.

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APPENDIX A:

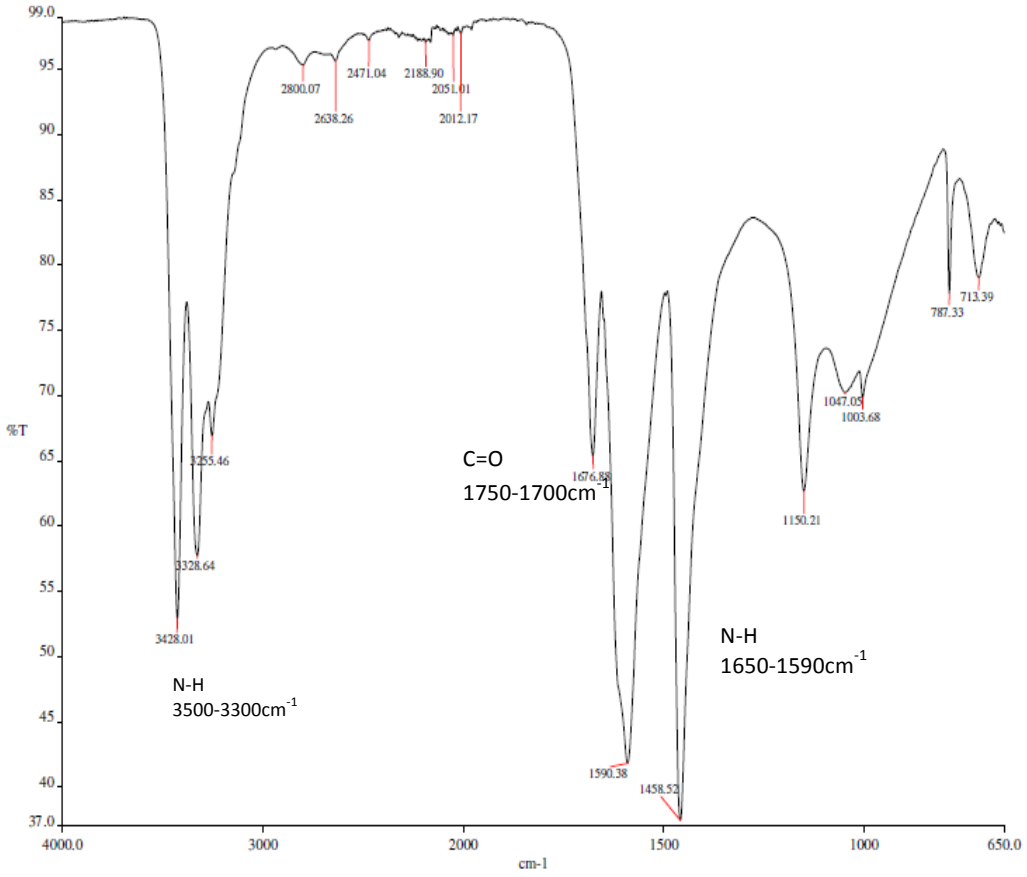

Figure A-1: IR of Urea (735nm)

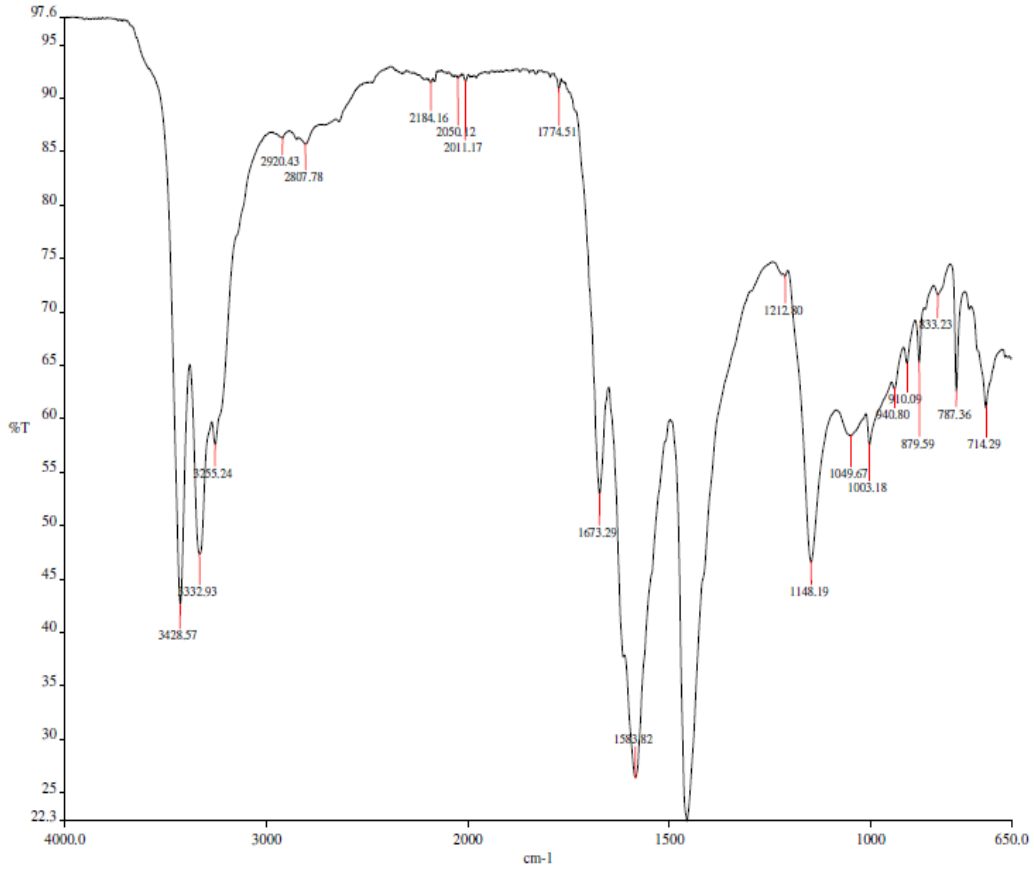

Figure A-2: IR of NaOH/urea

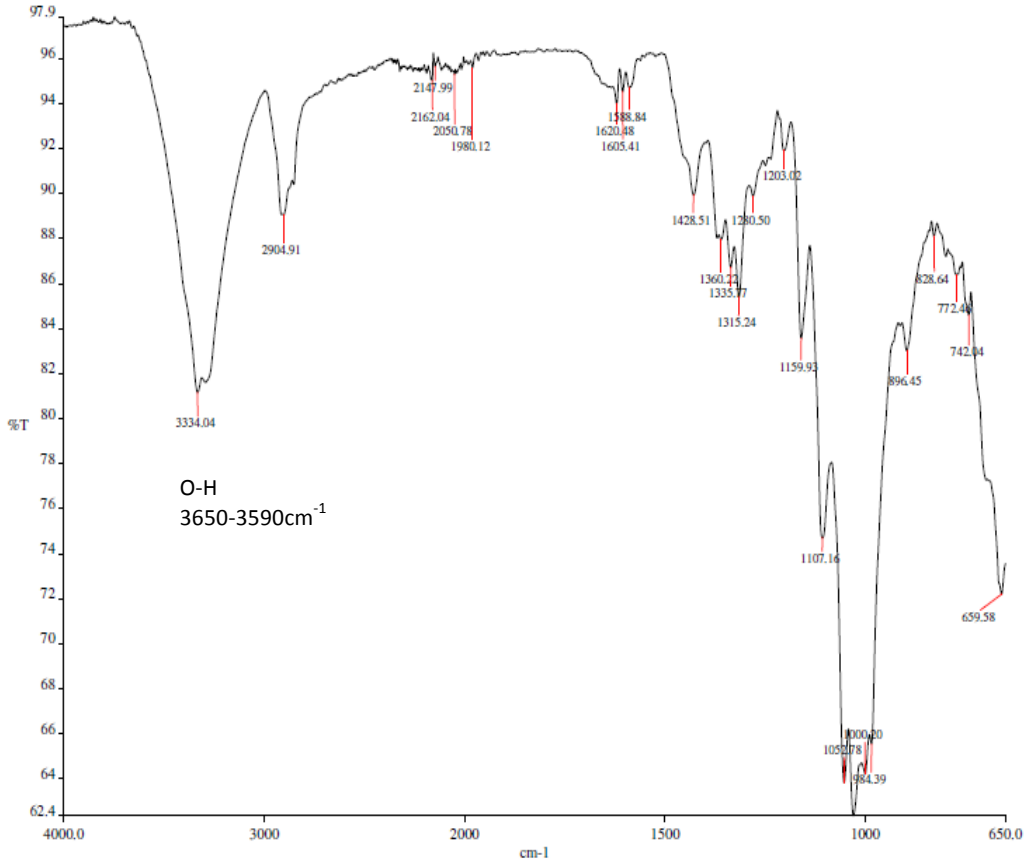

Figure A-3: IR for Non-treated Cotton Fiber

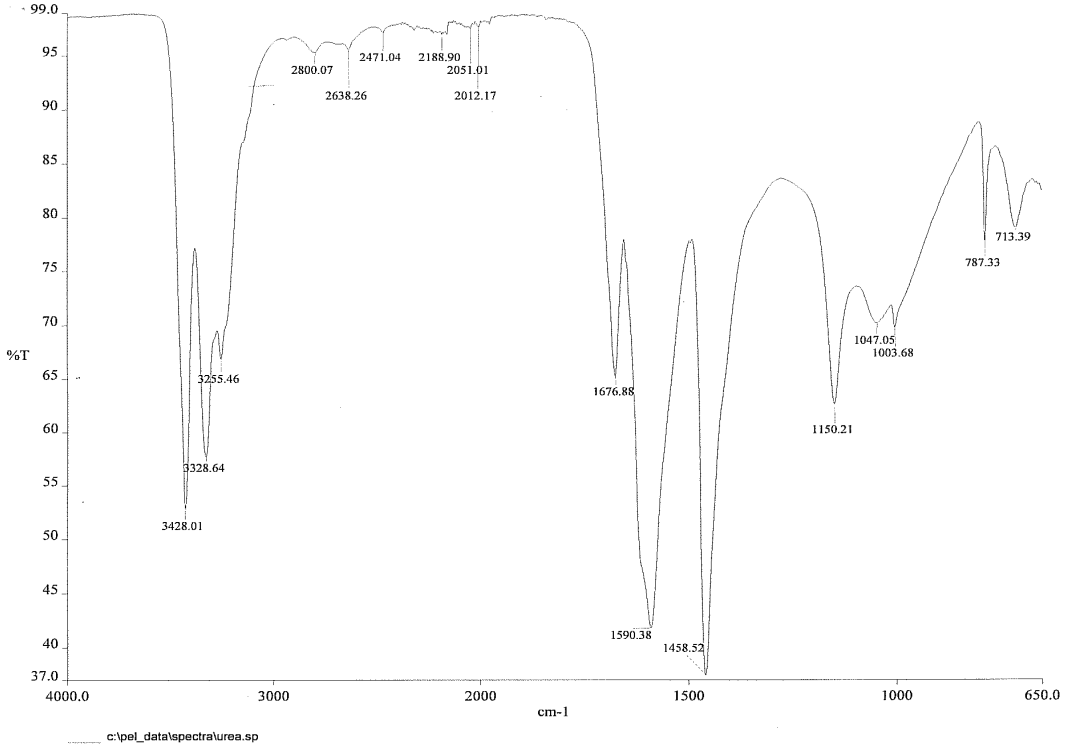

Figure A-4: IR of Pretreated Cotton Fiber with 7%NaOH/ 12%urea at -10ºC for 4mins

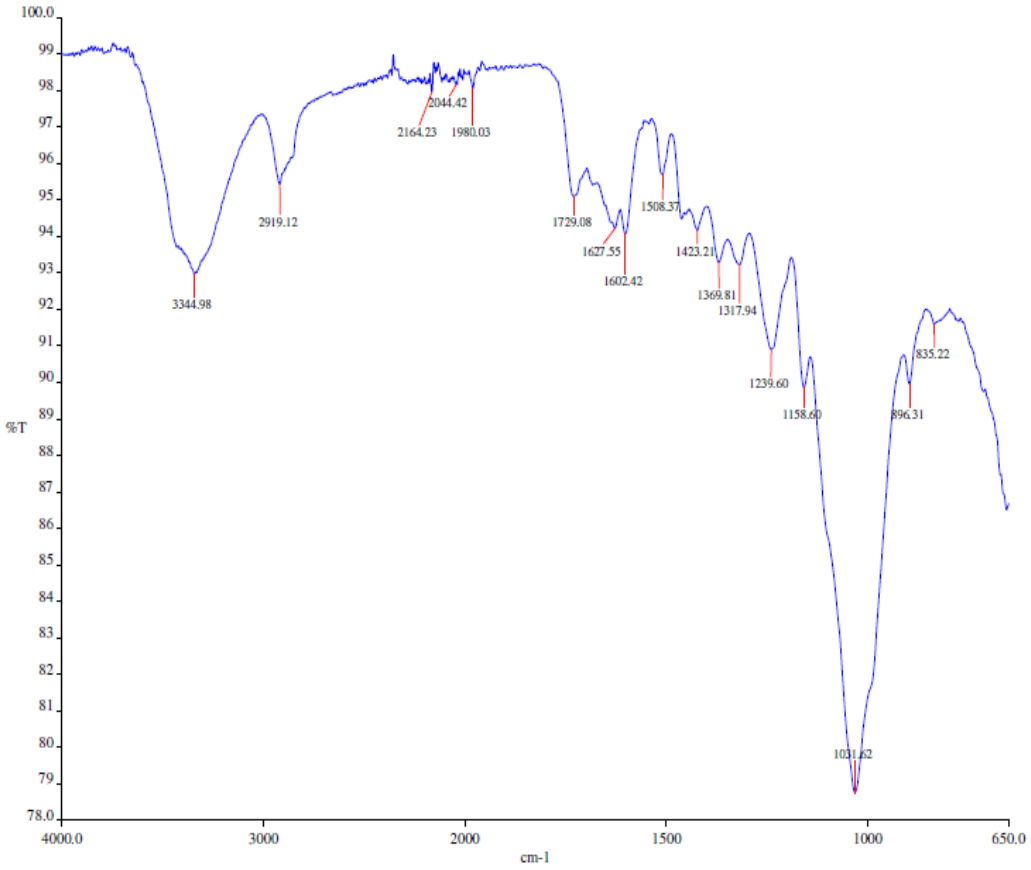

Figure A-5: IR of Non-treated Switchgrass Samples

APPENDIX B:

Table B: Descriptions of Enzymes Contained in the Novozymes Biomass Kit

NS22074 Cellulase Complex	This cellulase preparation catalyzes the breakdown of cellulosic materials into glucose, cellobiose, and higher glucose polymers. NS22074 can be used to reduce the viscosity or increase the extraction yield of various products of plant origin. The main reaction products of cellulose hydrolysis with NS22074 are cellobiose and glucose. Testing for synergy with NS50010 and NS22036 is recommended to maximize performance.
NS50010 β-glucosidase	β-glucosidase, also known as cellobiase, hydrolyzes cellobiose to glucose. cellobiose is a carbohydrate consisting of two molecules of D-glucose linked together by a β-1,4-glocosidic bond. NS50010 can be used to supplement NS22074 in order to increase the yield of fermentable sugars.
NS50012 Enzyme Complex	NS50012 is a multi-enzyme complex containing a wide range of carbohydrases, including arabinase, β-glucanase, cellulase, hemicellulases, pectinase, and xylanase. NS50012 can break down cell walls for the extraction of useful components from plant tissue and can be used in the processing of cereal and vegetable materials. The enzyme has the ability to liberate bound materials and can degrade a variety of non-starch polysaccharides.
NS22002 β-glucanase xylanase	NS22002 contains a mixture of β-glucanase and xylanase enzyme activities. β-glucanase and xylanase are the two main enzyme activities in the enzyme preparation, but the product also contains several other side activities, including cellulase, hemicellulase, and pentosanase.
NS22035 Glucoamylase	NS22035 is used to liquefied starch-containing substrates to produce sugars for fermentation. The enzyme preparations work in dedicated saccharification stages as well as simultaneous saccharification and fermentation operation. The glucoamylases hydrolyze both 1,4- and 1.6-alpha linkages to liberate glucose for subsequent fermentation by the yeast.
NS22036 Xylanase	NS22036 is a purified endo-xylanase with a high specificity towards soluble pentosans. NS22036 can be used to liberate pentose sugars from biomass hemicelluloses fractions.

Table C: Description of Activity Units[48]

Abbreviation	Description	Definition
CBU	Cellobiase Unit	One Cellobiase Unit (CBU) is the amount of enzyme that releases 2μmol glucose per minute under standard conditions with cellobiose as substrate.
EGU	Endo-Glucanase Unit	Endo-glucanase activity in EGU is measured relative to a Novozymes enzyme standard.
FBG	Fungal β-Glucanase Unit	One FBG (Fungal β–Glucanase Unit) is the amount of enzyme that produces reducing carbohydrate equivalent to 1μmol glucose per minute under the conditions given in the method. The activity is determined relative to an enzyme standard.
FXU-S	Fungal Xylanase Unit	Endoxylanase activity in FXU-S is measured relative to a Novozymes FXUS enzyme standard.
AGU	Amyloglucosidase Unit	Amyloglucosidase activity in AGU is measured relative to a Novozymes AGU enzyme standard.
PGU	Polygalacturonase Unit	Polygalacturonase activity in PGU is measured relative to a Novozymes enzyme standard.